TAMPEREEN TEKNILLINEN YLIOPISTO
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ADVANCED SURFACE TREATMENT OF ELASTOMERIC POLY-DIMETHYLSILOXANE FOR CELL STRETCHING APPLICATIONS

Master's thesis

Examiner: Professor Pasi Kallio

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Polydimetyylisiloksaani (PDMS) on elastomeeri, jota käytetään laajalti biologisissa dynaamisissa mikrofluidisissa sovelluksissa. Hydrofobinen PDMS ei kuitenkaan tue solujen kiinnittymistä viljelyn aikana, varsinkaan muuttuvissa olosuhteissa, kuten venytyksessä. Toisaalta PDMS ominaisuudet ovat soluvenytyssovelluksille liian hyödyllisiä, jotta se olisi helppo korvata. PDMS:n elastisuus, muovailtavuus, kemiallinen inerttisyys ja bioyhteensopivuus selittävät sen laajamittaisen käytön biolääketieteen alalla. Sen vuoksi PDMS:n pintakäsittely on välttämätön osa materiaalin käyttöä, varsinkin monimutkaisemmissa solusovelluksisa. Jotta kantasolujen käyttäytymistä ymmärrettäisiin paremmin, on tärkeää tutkia kestäviä pintakäsittelymenetelmiä.

Tämän diplomityön päätavoitteena oli etsiä menetelmiä, joilla pystytään sitomaan tyypin I kollageenia kovalenttisesti PDMS soluviljelyalustoihin pitkäaikaisia soluvenytyskokeita varten. Toissijaisena tavoiteena oli kehittää uusia paranneltuja pintakäsittelymenetelmiä. Työssä esitellään ja tutkitaan seitsemää eri pintakäsittelymenetelmää, joista neljä perustui uuteen tapaan käyttää askorbiinihappoa (AA) kollageenin sitomiseen. Menetelmiä tutkittiin käyttämällä immunofluoresenssivärjäyksiä ja soluviljelyä. Tehdyt kokeet on jaettu viiteen vaiheeseen. Ensimmäisessä vaiheessa (P1) physisorptioon pohjautuvat menetelmät ja glutaraldehydipohjainen Kovalenttinen Menetelmä 1 kuvattiin fluoresenssimikroskopian avulla. Toisessa vaiheessa (P2) uusi AA:n perustuva Kovalenttinen Menetelmä 2, kahtena eri versiona, kuvattiin myös fluoresenssimikroskopian avulla. Vaiheessa kolme (P3) fysisorptiomenetelmä, Kovalenttinen Menetelmä 1, sekä Kovalenttinen Menetelmä 2, kolmena eri versiona, testattiin viljelemällä rasvakudoksen kantasoluja (hAdSC) niiden päällä staattisesti 14 päivää. Vaiheessa neljä (P4) Kovalenttinen Menetelmä 1 ja Kovalenttinen Menetelmä 2, kahtena versiona, testattiin viljelemällä hAdSC:ja niiden päällä staattisesti ja dynaamisesti 13 päivää. Vaihe viisi (P5) esitteli uudentyyppisen Kovalenttisen Menetelmän 3, jota kuvattiin fluoresenssimikroskopian avulla, ja testattiin viljelemällä hAdSC:ja pinnoituksen päällä staattisesti neljä päivää.

Tyypin I kollageeni onnistuttiin kuvaamaan kaikissa pinnoitusmenetelmissä. Solujen viljely onnistui myös niin staattisessa kuin dynaamisessakin ympäristössä. Kokeiden tulokset osoittivat, että uusi AA ristisilloitettu Kovalenttinen Menetelmä 2 oli parempi sitomaan kollageenia, sekä sopivampi soluviljelyyn kuin physisorptiomenetelmä tai Kovalenttinen Menetelmä 1. Soluviljelykokeet tehtiin ja PDMS:n pintakäsittelymenetelmät kehitettiin osana innovaatiorahoituskeskus Tekesin rahoittamaa Ihmisen Varaosat hanketta ja Suomen Akatemian rahoittamaa WoodBone projektia.

ABSTRACT

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Polydimethylsiloxane (PDMS) elastomer is widely used in dynamic biological microfluidic applications. Hydrophobic pristine PDMS does not support cell attachment and culture, especially in dynamic conditions. Regardless, PDMS has too many useful properties as a base material for dynamic cell culture systems to be easily replaced. The good elastic properties, mouldability, transparency, chemical inertness, and biocompatibility of PDMS are enough to justify its use in large scale in the biomedical field. Therefore, PDMS surface treatment is nowadays considered as an essential step in using the material, especially for longer culture periods and dynamic culture conditions. To understand cell behaviour and stem cell differentiation better during cyclic stretching, it is important to study different durable surface treatment methods.

The primary goal of this thesis work was to covalently bind collagen type I to cell culture substrates fabricated from Sylgard® 184 PDMS composite for long term cell stretching experiments with methods found in the literature. The secondary goal was to propose a novel surface treatment method to improve upon the existing methods. Seven different surface treatment methods, four of which were novel ascorbic acid (AA) based methods, were studied in this thesis using immunofluorescent imaging and cell culture experiments. The experiments were divided in five phases in chronological order to reflect the evolution of the surface treatment methods and the experiments. In phase one (P1), physisorption and Covalent Method 1 were imaged using fluorescent microscope. In phase two (P2), a novel Covalent Method 2 in two variations was proposed and subsequently imaged using fluorescent microscope. In phase three (P3), physisorption, Covalent Method 1, and Covalent Method 2 in three variations were tested in static human adipose stem cell (hAdSC) culture for 14 days. In phase four (P4), Covalent Method 1 and Covalent Method 2 in two variations were tested in static and dynamic hAdSC culture for 13 days. Phase five (P5) introduced Covalent Method 3 that was imaged with fluorescent microscope and tested in static hAdSC culture for four days.

Collagen type I was successfully labelled and imaged from all of the coatings. Cells were also successfully cultured in static and dynamic environments. The results showed that the novel AA crosslinked Covalent Method 2 was superior to the physisorption method and Covalent Method 1 in immobilizing collagen as well as in cell culture tests.

The cell culture tests were conducted and PDMS surface treatment methods were developed for Human Spare Parts project funded by Tekes, the Finnish Funding Agency for Innovation and WoodBone project funded by the Academy of Finland.

FOREWORD

This thesis work was conducted in Micro- and Nanosystems Research group of BioMediTech Institute and Faculty of Biomedical Sciences of Tampere University of Technology in collaboration of Human Spare Parts and WoodBone projects, and was supervised and examined by the group leader professor Pasi Kallio. Many researchers from the group and the Institution assisted me in the experiments along the years leading to this thesis including Joose Kreutzer, Juha Hirvonen, Feihu Zhao, Marlitt Viehrig, Sanni Virjula, Anna-Maija Honkala, Lassi Sukki, Samu Hemmilä and many others in small but significant assisting roles.

I want to emphasize my gratefulness to Joose Kreutzer and Professor Pasi Kallio for taking my application and me under review, and then accepting me into the group as a summer trainee in 2012. This marked the inception of my ongoing scientific career, which also directly led to this thesis work and topic.

Cell experiments included in this thesis were conducted by Sanni Virjula assisted by Anna-Maija Honkala in Adult Stem Cell Group led by docent Susanna Miettinen of BioMediTech Institute and Faculty of Medicine and Life Sciences of University of Tampere. The cell stretching devices used in this study were made according to the published designs by Joose Kreutzer. He also provided invaluable technical help in setting up, optimizing, and using the stretching system.

Finally, I want to thank my ever loving and understanding wife, Susanna, and our wonderful miniature schnauzers Roope and Pyry, for keeping me sane during the tough process that resulted in this thesis. I am extremely grateful for your love and support!

Tampere, 20.5.2017

Joni Henrik Gustaf Leivo

"Hope for the best, yet do none in jest.
Prepare for the worst, though not headfirst.
Expect the average, but lose not your leverage.
I say,
follow this rule.
You shan't find yourself
under ridicule."

J.L, 2017

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AA	L-ascorbic acid
ABS	n-4-(azidobenzoyloxy)succinimide
ASC	Adult stem cell
APTES	(3-aminopropyl)triethoxysilane
COGA	Coating with glutaraldehyde immobilized collagen I
COAA1-3	Coatings with ascorbic acid immobilized collagen I 1-3
COGEL	Coating with ascorbic acid immobilized collagen gel
CSD	Cell stretching device
CVD	Chemical vapour deposition
DI water	Deionized water
DPBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
ESC	Embryonic stem cell
GA	Glutaraldehyde
hAdSC	Human adipose stem cell
iPSC	Induced pluripotent stem cell
MSC	Mesenchymal stem cell
NHS	N-hydroxysuccinimide
PAA	Polyallylamine
PDMS	Polydimethylsiloxane
P1 – 5	Experimental phases 1 – 5 of the thesis study
PHY1-2	Coatings with physisorbed collagen type I 1-2
PS	Polystyrene
RGD	Arginine-glysine-aspartic acid
Sulfo-SAND	Sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)ethyl-3-dithio-propionate
Sulfo-SANPAH	Sulfosuccinimidyl-6-(4-azido-2-nitrophenylamino)hexanoate

1. INTRODUCTION

Cell culture techniques have evolved rapidly during the last few decades. What started as a simple two-dimensional culture on a simple plastic or glass plates can today be a complex system with not only controlled temperature and humidity but also controllable dynamics and chemistry of the substrate or the culture medium. Today, researchers routinely grow cells on three-dimensional (3D) scaffolds (Chevallay, Herbage 2000, Tirkkonen, Haimi et al. 2013), in multi-cell co-cultures (Goers, Freemont et al. 2014), and dynamic culture systems (Leung, Glagov et al. 1977, Lee, Delhaas et al. 1996, Wipff, Majd et al. 2009, Ahmed, Kural et al. 2010, Majd, Quinn et al. 2011, Figueroa, Kemeny et al. 2011, Zhao, Zhou et al. 2011, Kreutzer, Ikonen et al. 2013, Ugolini, Rasponi et al. 2016) *in vitro* to mimic the natural habitat of the cells. Dynamic culture systems often exploit microfluidic principles or microfabricated substrates along with rapid prototyping to create versatile controllable platforms for various cell culturing needs.

Single cells are often viewed as rather passive creatures that mostly consume and proliferate. If we take a look at native tissues, however, it becomes obvious that cells are active sensing beings that react to not only chemical and biological, but also physical cues. For example, in our tissues muscle cells and bone cells are affected by constant forces in various directions. They are also necessary for the healthy growth of these tissues. As researchers' interest in this topic increased, it eventually grew into a completely new field of study. Cell stretching is one of the older concepts in this field (Leung, Glagov et al. 1977) that focuses on physical stretching of cells *in vitro* to study and control cell behaviour. For this reason, biomedical engineers focus on creating devices that can mimic these forces *in vitro*. Ultimately, the aim is to create culture systems with conditions closer to native tissues, and to fully integrate measurement components as basic parts of the full system. Eventually, this can lead to the control over cell fate, and to even growing fully functional tissues in a laboratory environment.

Polydimethylsiloxane (PDMS) based elastomers are one of the most widely used silicone materials for constructing devices for a wide range of biomedical applications (Berthier, Young et al. 2012), although it is especially useful for cell stretching applications. It is often chosen as the substrate and also the device material which is in direct contact with tissues, cells and biological fluids, a sign of its versatility as a material. PDMS that is used in biomedical devices is a silicone elastomer with controllable rubber-like elasticity, glass-like transparency, and it is non-hazardous to any cells growing on the material. Nowadays, rapid prototyping with the material in laboratories worldwide is a common practice that requires no special facilities. PDMS can be permanently bonded to itself, glass or polystyrene (PS) after a simple plasma treatment, enabling the creation of surprisingly complex structures that are seamless and adhesive free.

The main drawback of PDMS in biomedical applications lies in its surface properties. While it is technically non-hazardous to cells, PDMS surface is highly hydrophobic and completely unsuitable for cell adhesion in its native state. However, by exploiting the chemistry at the PDMS elastomer surface, the situation can be critically improved. A common practice is to functionalize PDMS with extra cellular matrix (ECM) proteins before using it as a cell culture substrate. Different plasma, physical, chemical, and more complex advanced surface treatment methods have been used to improve the suitability of PDMS substrate for the cells. Additional challenges are brought by the dynamic culture, especially cell stretching, as the physically strained substrate can easily lose hold of the coating and along with it the cells. Step-by-step and layer-by-layer chemical treatments aimed at covalent immobilization of ECM proteins have been created to circumvent the disadvantages that basic physical adsorption has. However, the complexity of such treatments raise highly relevant questions about the effects these types of treatments can have on different types of cells. A massive amount of basic research is needed in this field to propose more durable and biocompatible alternatives for current treatment methods. Furthermore, cell culture experiments using different cells and different ECM proteins are necessary to create a bigger picture about the cues leading to stem cell differentiation, or just to propose the optimal parameters for complex cell culture systems. One must bear in mind that dead cells tell no tales.

All of the concepts mentioned above are visited in the theoretical part of this thesis. Chapter 2 presents cell stretching as a concept and introduces the reader to the cells and tissues relevant to the field. Mechanically active tissue types and ECM are presented along with the concept of stem cells and differentiation. In the end of Chapter 2, a set of cell stretching studies and devices is introduced. Chapter 3 provides a thorough introduction to PDMS as a material and its properties. Furthermore, basic PDMS surface treatment methods are presented and explained. Finally, Chapter 3 ends with a literature survey of the most relevant advanced surface treatment methods that have been recently used to modify PDMS.

The aim of this work is to find suitable coating techniques for the use in the pneumatic cell stretching devices (CSD) made of PDMS and glass as described by Kreutzer *et al.* (Kreutzer, Ikonen et al. 2013) and study them under the fluorescent microscope. The main aim is to find and implement coating methods from literature that withstand the stretching caused by the device in normal cell culture conditions and that can be utilized without special equipment. A secondary aim is to propose a novel surface treatment method for implementation. In the experimental part of this thesis, seven different coating methods for PDMS are proposed, implemented and studied. In Chapter 4, the experimental set up and the experiments are thoroughly explained. In this work, Collagen type I ECM protein is adhered or bound to the PDMS CSDs using physisorption or covalent immobilization via crosslinker molecules. These treatment methods are studied in their ability to bind collagen type I to the PDMS surface and support long term static and dynamic hAdSC

culture. Chapter 5 presents the results from the experiments in five distinguishable phases in chronological order. The thesis work is concluded in Chapter 6.

THEORETICAL PART

2. CELLS AND CELL STRETCHING

The concept of cell stretching has been a target of studies for a long time (Leung, Glagov et al. 1977), but only recently, it has started to spark more interest in a wider range of research groups. While it has been common knowledge for a while now that skeletal, vascular and heart muscle cells can feel strain and stretch, and bone and cartilage cells compression, the main interest in dynamic cell culture studies has been focused towards stem cells. Today, as stem cells are rather easy to harvest, isolate or induce, and overall to get a hold on, the interest is to achieve full control over the cells' differentiation path in the hope of creating certain cell types. In the future, these techniques could be used in creation of functional natural tissues, such as bones, muscles or heart in the confinement of a laboratory from the patient's own cells.

This Chapter will bring forth relevant information about cells, tissues and ECM regarding mechanical stimulation studies and then move on to stem cells and differentiation. Furthermore, the Chapter will survey some of the recent studies in the field of cell stretching and describe different methods for applying stretch to cells.

2.1 Cells and tissues for mechanical stimulation research

A cell is the fundamental building block of all life. From the smallest of bacteria to largest of sea mammals the cell is the smallest, and in the case of bacteria, only, functional unit in a living organism. There is a wide variety of different cell types, but only some of them are relevant in cell stretching studies. Cells living in physically moving tissues make the most obvious targets in mechanical stimulation or stretching research.

Muscle tissue has the ability to convert energy into contractive movement. Muscle cells contain special filaments which consist of proteins myosin and actin. When actin slides past myosin, the ends of the filaments move closer to the centre resulting in contraction. Skeletal muscle tissue found in voluntary muscles, cardiac muscle tissue found in the heart and smooth muscle tissue found in blood vessels, stomach, and intestines all function in similar fashion by utilizing myosin and actin filaments. All muscle cells are thus affected by stretching in their native environment. Heart cells, especially, are under much interest due to the amount of heart disease today, and the limited regenerative capabilities of the heart tissue. Creating functional beating heart tissue from stem cells and seeding them into a heart scaffold (Guyette, Charest et al. 2016) could make a difference for millions of people every year. However, culture systems and complex mechanisms of cellular differentiation must be opened first to create tissues that are truly comparable to native tissues.

Connective tissue is a broad term for tissues, ligaments and fluids that connect organs and other parts of the body. Bone is probably the most recognized part of connective tissue, but there are other types that are interesting for mechanical stimulation research. Anyhow, bone tissue encounters strong mechanical forces in its native environment and is built to withstand strong compressive forces. In comparison to muscle tissue, it functions in a completely different way. Bone tissue, or the bone cells within the hydroxyapatite mineralized collagen matrix, reacts to compression and lack of thereof instead of actively producing mechanical stress themselves. Cells called osteocytes live within bone matrix in isolation, albeit interconnected to other osteocytes by long processes. They control the bone forming osteoblasts and bone breaking osteoclasts via the mechanical cues carried by the bone matrix to optimize the tissue strength for the specific location. Osteocytes mature from osteoblasts that get isolated from other osteoblasts and surrounded by collagen matrix. It would be important to know if mechanical cues play a role for stem cells, which are immature cells with no specialization, to differentiate into specialized bone cells, for example. Creating functional bone tissue from stem cells in a reliable large scale way would be an important step towards modelling and curing of diseases of bone and could accelerate the healing of the injured bone of accident victims. Bone grafts with differentiated and specialized cells are much safer than the stem cell based equivalents. (Bonewald, Johnson 2008)

Cartilage is flexible tissue that usually covers bone tissue at joints. Cartilage is also found in the spine between vertebrae, bronchial tube, ribs, ears and nose. Cartilage consists of mostly ECM of proteoglycans and collagen type II. Similarly to bone, the ECM surrounds the developing cells in the growing matrix and leaves them in isolation. These become chondrocytes that slowly increase the amount of ECM around the cells. Due to the nature and location of cartilage tissue, it lies under varying strong compressive mechanical forces. The abundant elastic elastin protein fibres help in absorbing the forces, while the chondrocytes sense the mechanical stress and react to it actively by producing more ECM proteins and proteoglycans (Grodzinsky, Levenston et al. 2000). Knowing this and the fact that cartilage tissue is very slow to repair and regenerate after injury, cartilage tissue is a prime candidate for mechanical stimulation studies. Implanting stem cells on cartilage injuries to help repair the tissue has already been studied, but with mediocre results, a telltale sign of the inherent complexity of the functionality of cartilage tissue. Maturing stem cells before implantation with mechanical stimulation could be the next logical step in implementing this technology. However, the problem of the lack of vascularization in cartilage tissue impedes the more simplistic regeneration attempts; therefore, more complex approach might be necessary. (Huey, Hu et al. 2012)

The loose and dense types of fibrous connective tissues also encounter mechanical forces in the body. Loose connective tissue, which is mainly known for adipose and areolar tissues, fills the space between organs and keeps them in place. They consist of loosely interconnected collagen type I and elastin fibres, and sparsely distributed fibroblasts, cells

that create the ECM proteins for expanding and repairing the tissue. Dense connective tissue, such as tendons and ligaments, consist of densely packed ECM of collagen type I fibres with varying amounts of proteoglycans and elastin. Both tendons and ligaments also have a small amount of cells residing among the fibres. Most of these cells are specialized fibroblasts that make and repair the ECM, but there are also reports of isolating stem cells from ligaments (Cheng, Liu et al. 2010). Tenocyte, which is a special type of fibroblast distributed in tendons, has been shown to sense mechanical cues which make them also a prime target for tissue engineering and mechanical stimulation research (Schiele, Marturano et al. 2013). Tendons and ligaments both lack blood vessels, so their repair rate after injury is very slow, similarly to cartilage. While their composition is rather simple and well defined, they are prone to injuries, because of their function and position in locations known for strong physical forces. For this reason, artificial tendons and ligaments as spare parts are constantly studied (Cheng, Liu et al. 2010, Scott, Danielson et al. 2011, Schiele, Marturano et al. 2013, Yang, Rothrauff et al. 2013), but much work is still needed to be able to grow e.g. tendons in a laboratory. Stretching applications could help in achieving information about the differentiation cues needed for tenocyte culture or providing mechanical load for the ECM and the cells to get closer to the natural mechanical integrity of the tendons, for example. Knowing also what type of stimulation or stretch causes stem cells to take the differentiation path into fibroblasts, can help researchers to avoid this type of stimulation as other cell types of mesenchymal origin such as bone, cartilage, or cardiac cells are often preferred.

2.2 Extra cellular matrix

ECM is an integral part of most tissue types in multicellular organisms. It is a mesh, network or a sheet of molecules that provide a support structure for the body and its tissues and the main component of connective tissues. The composition of ECM and its function changes depending on the location and the needs of the surrounding tissues.

ECM literally translates to a “matrix outside of the cell” and it is basically filler to the space that is not occupied by cells. It is created and secreted by the cells that reside in it as a supporting structure to provide mechanical durability and a substrate to the nimble cells of the surrounding tissues. In general, ECM types can be divided between collagen-dense fibrous matrices and sheets, and polysaccharide-dense negatively charged gels. The blood plasma is also a type of ECM but it is not further discussed here. Depending on the type, location, and function, the ECM can consist of fibrous proteins collagen and elastin, special proteins and glycosaminoglycans or proteoglycans, which are huge complexes of proteins and glycosaminoglycans.

Fibrous proteins dominate in connective tissues and their main component is collagen, which is also the most abundant protein in the human body. Collagen fibrils and bundled fibrils called collagen fibres provide most of the tensile strength of human tissues. They are created, bundled together and interwoven into a strong matrix by fibroblasts in most

tissues, by osteoblasts in bone and by chondroblasts in cartilage. Tendons and ligaments are basically aligned fibres of collagen attached to the bones and muscles. Elastin provides the elasticity required by some tissue types. It is responsible for returning the original shape of skin, lungs and blood vessels, for example, after deformation. (Alberts, Bray et al. 2010)

The empty spaces between cells and fibrous ECM is filled by a gel of proteoglycans, glycosaminoglycans and proteins. While proteoglycans come in a myriad of different conformations they are typically glycosaminoglycans linked to a core protein that is in turn linked to another glycosaminoglycan chain. These complexes are huge space fillers and can be as large as a bacterial cell. Their negative charge attracts cations, such as Na^+ and K^+, which are plentiful in the blood plasma and extracellular fluid. These cations are osmotically active and they cause the formed gel to absorb water many times its own weight. The swelling pressure caused by this is utilized by many tissues to withstand pressure. When combined with collagen matrix, the ECM can withstand enormous pressure from the inside and from the outside, as can be witnessed in the cartilage tissues of joints. The proteoglycan ECM gel can vary in pore size and guide or block cell migration and differentiation, regulate passage of signalling factors or bind growth factors, all in addition to providing hydrated space around cells. These molecules, as with every ECM related component, are created and secreted by cells residing in the immediate area. (Alberts, Bray et al. 2010)

Cells bind to the ECM via proteins called integrins. Integrins are small two part proteins that attach to the cytoskeleton with one end and to the ECM with the other end that sticks out of the plasma membrane. Signals from the cytoskeleton and vice versa can be transmitted via this interaction between the cell and the ECM. Integrins can attach the cell directly to the ECM networks or indirectly by binding secondary binding proteins such as fibronectin. Fibronectin is an important protein that is able to bind collagen fibrils. Cells attach to bare collagen poorly, which is why fibronectin is a necessary intermediary between the cell and collagen. Cells cannot attach or crawl over collagen unless fibronectin is present. (Alberts, Bray et al. 2010)

The cells have an undeniable relationship with all ECM types. For this reason, almost all biomedical technologies utilizing cells must also utilize ECM components. Nowadays it is understood that many of the critical reactions that cells feel and go through can be manipulated by a proper use of ECM components. One piece of evidence about this was the discovery of the so-called arginine-glysine-aspartic acid (RGD) tripeptide sequence. This RGD-peptide is recognized as a binding site by many integrins. Synthetic RGD-peptide treated surfaces or materials, for example, can be made recognizable to cells as the integrins naturally bind to RGD sequence: cell attachment can be manipulated or molecules like drugs made bondable to cells without any complete ECM component (Ruoslahti 1996). It is, however, more common to use complete proteins to functionalize materials for implantation or cell culture purposes. Proteins such as collagen type I and

type IV, laminin, and fibronectin are widely used, as is proteoglycan hyaluronic acid. These ECM molecules are easy to extract from tissues and are readily available for sale. They often behave in predictable manner in appropriate solutions, which makes their use straightforward. Collagen type I, and the other types, can self-assemble into fibrils and fibres after being extracted and dissolved in an acidic solution (Pins, Christiansen et al. 1997). In physiological conditions, the broken collagen molecules form natural fibre matrix that cells can adhere and grow on. If there is enough collagen it can form even a hydrogel or porous sponge which can be used as a 3D scaffold in cell culture and other biomedical applications (Chevallay, Herbage 2000). Furthermore, when combined with proteoglycans or hyaluronic acid the gel or sponge scaffold can become more rigid, hydrophilic, and resistant to dissolution as explained by *Davidenko et. al.* (Davidenko, Campbell et al. 2010). Laminin, a key protein component of basal lamina of the basement membrane, is also used to form cell adhesive networks on culture substrata, as is glycoprotein fibronectin. They can both, along with hyaluronic acid, interact with collagens to create even more complicated ECM networks. Finding the optimal combination of ECM molecules and the resultant physical properties of 2D or 3D matrix for different applications is one of the biggest challenges of biomedical research and it lies at its spearhead. Engineers need to find ways to combine the knowledge from cell biologists and biomedical engineering to incorporate biomolecules from ECM into their applications.

2.3 Stem cells and differentiation

Stem cell is a special type of cell with two characteristic properties: They can make identical copies of themselves, and form other cell types via the process of differentiation. They act as the mothers of all other cells and with the ability of self-renewal they can be potentially immortal. Overall, stem cells hold most of the potential in cell based applications, thus they are the star players in the current biomedical research.

2.3.1 Stem cell basics

During the cell division of a stem cell the formed daughter cells have two options: They can keep their stem cell phenotype and continue multiplying and act as a source of immature cells, or they can take the path into differentiation and specialized cell types. The first option is called self-renewal and it gives the stem cells their ability to be a potentially unlimited source of cells. In adult tissues, stem cells control the self-renewal process via chemical, electrical and mechanical cues to only happen when needed and to produce the right type of progeny. Being in control of self-renewal process is crucial as any rogue cells multiplying out of control can quickly form tumours (Erdö, Bührle et al. 2003). This also counts implanted stem cells. Lack of control is still one of the issues regarding technologies dependent on implanted stem cells (Erdö, Bührle et al. 2003, Pittenger, Kerr 2015), and it critically limits their immediate clinical significance. Stem cells are generally divided into embryonic, adult, and induced stem cells depending on their source.

Embryonic stem cells (ESC) can be found in the developing embryo and they have the widest differentiation potency of the cells that are relevant for research. Adult stem cells (ASC) are found in adult tissues and they maintain and repair healthy tissue, and regenerate and heal damaged tissue. Induced stem cells are artificially created or induced from harvested specialized cell types. (Pittenger, Kerr 2015)

Stem cell differentiation capability can be organized through the concept of potency. Stem cells are totipotent, pluripotent, multipotent, oligopotent, or unipotent depending on the number of tissue types they can create. Totipotent stem cells can create any cell for the entire organism, including the embryo's supporting tissues placenta and umbilical cord. Only the first few cell divisions after the fertilization of the oocyte are totipotent, thus they are rarely used for research purposes, even though they have the ultimate differentiation capacity. Pluripotent stem cells can create any tissue of all three germ layers endoterm, mesoderm and ectoderm, but not placenta or umbilical cord. For research purposes, pluripotent stem cells can be harvested from the inside of a blastocyst and brought to culture as ESCs, and renewed indefinitely. While ESCs could be regarded as one of the most valuable cell types for research due to the infinite differentiation potential, in reality, they are quickly losing relevance in the studies of the day, due to the difficulties in the acquisition of these cells. Furthermore, there was a significant underlying ethical issue in harvesting ESCs from available embryos. Today, ESCs are created *in vitro* by fertilizing donated eggs. While the process is not ethically as troublesome as harvesting embryos *in situ*, the efficiency of the *in vitro* processes is quite low. Those points combined have led to the favouring of other strategies in stem cell studies, such as induced pluripotency, or harvesting multipotent stem cells from adult individuals. Multipotent stem cells have already dedicated themselves into a specialized role. These cells have the capacity to differentiate into multiple, but not all, cell types. They are sometimes called progenitor or precursor cells. They still have the ability for self-renewal and keeping their progenitor or stem cell state. Mesechymal stem cell (MSC) that can form cartilage, bone, muscle and fat tissues, is an example of a multipotent stem cell. There are also oligopotent stem cells that can create two or more cell types and unipotent stem cells which can create cells from single lineage only. The neural progenitor cell that can create cells of the neural system is an example of an oligopotent stem cell and the progenitor cell that creates the male sperm cells is a unipotent stem cell. (Pittenger, Kerr 2015)

Differentiation from stem cell state to specialized functional tissues is not a simple one step process. In fact, it often involves multiple sequences of cell division and sensitive evaluation of internal and outer influences. During this process, the expression of some genes deactivate, while some activate. The combined effect dictates what type of cell will be the final result. A signal that causes differentiation can be a change in basal nutrients, change in the cell's environment, stimulation or lack of thereof, introduction of a signalling molecule such as growth factor, a new cell-to-cell interaction, or loss of such, for instance. In the body, the stem cells reside inside so called stem cell niches, where they

can keep their replicating phenotype indefinitely (Scadden 2006). The niche can be a physical structure limited by ECM, or a habitat of stem cells flanked by other types of cells (Scadden 2006). A stem cell can replicate either symmetrically where it produces two identical daughter cells, or asymmetrically where it produces two nonidentical daughter cells. During self-renewal the cells, or at least one of them, get to keep their potency, in other words they stay inside the niche of their parent cell. The niches are usually structured in such way that only certain number of cells fit in while others are forced out (Scadden 2006). In the end, this forces asymmetry between the progeny of the parent cell as the other begins differentiation due to the change in the environment. Sometimes the asymmetry is the product of unequal distribution of cell organelles, or other cell fate determinants that can also force the daughter cell out of the niche, and cause the initiation of its differentiation. Stem cells may leave their niche to differentiate also without replication, a strategy commonly used for harvesting bone marrow stem cells (Broxmeyer, Orschell et al. 2005). After leaving the stem cell niche, the cell enters into a series of symmetric divisions that amplify the cell number, most notably in developing tissues or *in vitro* (Morrison, Kimble 2006). This stage is called transit amplification stage and the dividing cells transit-amplifying cells; these cells are progenitors that have abilities somewhere in between stem cells and fully differentiated cells and are often identified by their potency and potential progeny (Figure 1). *In vitro* the transit amplification phase can quickly lead to confluence and the need of passaging the cells into a subculture until the final number of cell divisions is reached (Uzgare, Xu et al. 2004), and the cells terminally differentiate into their final form. (Pittenger, Kerr 2015)

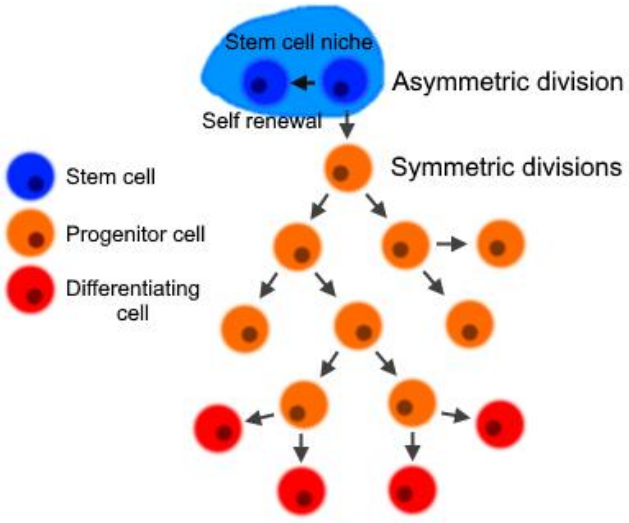

Figure 1. *A depiction of the development of a differentiated cell population that descends from a stem cell capable of self-renewal. Edited from (Pittenger, Kerr 2015)*

2.3.2 Adult stem cells

ASCs refer to cells that have capabilities for self-renewal and differentiation, but can be found in fully developed adult tissues. These cells are used by the body for regeneration and repair of aged and damaged tissues, and as a reservoir of new cells (Weissman 2000). ASCs are not pluripotent, however, so their differentiation capacity is more restricted, as they cannot produce every cell type. Rather, the body hosts a multitude of multipotent ASCs which are generally limited to produce the cell types of their home tissue. From the research standpoint, they can still offer advantages over pluripotent stem cells; harvesting has less ethical concerns, because every patient is a source of immunocompatible autologous stem cells that can be expanded and differentiated *ex vivo* (Pittenger, Kerr 2015).

ASCs were first discovered in bone marrow in the 60s in the form of hematopoietic stem cells (Till, McCulloch 1961), which develop into blood cells, and mesenchymal stem cells (Friedenstein, Chailakhjan et al. 1970) that can develop into bone, cartilage and muscle cells. After the first discoveries and the inevitable realization of the source of human's intrinsic tissue regeneration capabilities, stem cells have been found and isolated from many adult tissues; epidermal stem cells from epidermis (Lavker, Sun 1983), neural stem cells from brain (Carpenter, Cui et al. 1999), muscle stem cells from skeletal muscle (Baroffio, Bochaton-Piallat et al. 1995), lung stem cells from lung (Kajstura, Rota et al. 2011), intestinal stem cells from small and large intestine (Potten, Loeffler 1990), olfactory stem cells from nasal neuroepithelium (Roisen, Klueber et al. 2001), testicular stem cells from testicles (Conrad, Renninger et al. 2008) and MSCs or MSC like cells from many tissues such as skeletal muscle (Bosch, Musgrave et al. 2000), adipose tissue (Zuk, Zhu et al. 2001), dental pulp (Gronthos, Mankani et al. 2000), skin dermis (Young, Steele et al. 2001), bone periosteum (Nakahara, Goldberg et al. 1991), blood circulation (Chong, Selvaratnam et al. 2012) and walls of the peripheral vascular system (Covas, Panepucci et al. 2008).

ASCs are usually considered to be multipotent as they only produce cells of distinct lineage. On some rare cases, ASCs have been reported to have pluripotency (Roisen, Klueber et al. 2001, Jiang, Jahagirdar et al. 2002, Wagers, Weissman 2004, Guan, Nayernia et al. 2006), but more research is needed before that can be taken as a well-founded fact. However, there is evidence, where tissue based ASCs of distinct niche can produce wider range of progeny than previously thought. Migration to different tissue types and transdifferentiation is a real ability of these stem cells. MSCs have been shown to transdifferentiate into neural progenitor cells and show marks of astroglial and neuronal phenotypes (Ries, Egea 2012). Being readily available for harvest in adult bone marrow and adipose tissues, MSCs might have potential for neural system regeneration among their potential for connective tissue and muscle regeneration capabilities. Furthermore, neural stem cells have shown capabilities for differentiation into hematopoietic lineage (Bjornson, Rietze et al. 1999), while in turn according to some reports hematopoietic stem cells have shown

capabilities for differentiation into cardiomyocytes (Orlic, Kajstura et al. 2001) and neurons (Mezey, Chandross et al. 2000). However, the factors behind transdifferentiation are possibly even more complicated than those with regular differentiation. In another study, the cardiac differentiation of hematopoietic stem cells was specifically studied but no evidence of it could be observed (Murry, Soonpaa et al. 2004). Today, the factors behind natural stem cell transdifferentiation are still largely shrouded in mystery, but big steps have been taken to understand the apparent reprogramming of the cell phenotypes, and how it can be induced at least *in vitro*, if not *in vivo*. All in all, ASCs have been taken as an integral part of today's regenerative medicine doctrine. The availability and lack of ethical concerns in harvesting certain types of ASCs, such as adipose tissue derived MSCs, give an indefinite source of material for researchers to study differentiation, and to optimize methods for the regenerative cell therapies of the future.

2.3.3 Induced pluripotent stem cells

A quite recent advancement in stem cell technologies is the induced pluripotency of adult cells that otherwise lack any abilities of a stem cell. The obvious advantage of pluripotent stem cells over the less potent ASCs is the capability to produce any mature cell type. The ethical concerns regarding the harvesting of ESCs from human embryos, and the inefficiency of in vitro fertilized eggs, however, have largely inhibited the wider use of these cells. The vacuum left behind by this mismatch of supply and demand provided the impetus for finding alternative sources of pluripotent cells. Today, we know that there are potentially pluripotent ASCs that can be harvested from nasal cavity (Roisen, Klueber et al. 2001) or testes (Conrad, Renninger et al. 2008) without previously mentioned ethical concerns. In addition to these the induced pluripotent stem cell (iPSC) is another promising alternative to ESCs without the ethical or some of the immunological issues the use of ESCs usually has.

The genome of the ESCs and all of their progeny are the same in the same individual. This means that there are intrinsic factors that cause stem cells to be able to differentiate and also factors that cause mature cells to function in their special way. In their 2006 published study, Takahashi and Yamanaka (Takahashi, Yamanaka 2006) were able to convert differentiated fibroblasts into pluripotent ESC-like cells by retroviral infection of several transcription factors and oncogenes which were known to play role in the cells of an early embryo. Since then, the methods for iPSC generation have been extensively documented, tested for human cells, and polished for efficiency. Still, the efficiency of converting adult cells to iPSCs is 0.001-10 % at best and it is dependent on the cell source where the less differentiated cells usually convert to iPSCs more efficiently (Yu, Vodyanik et al. 2007). To utilize iPSCs in regenerative medicine to their full potential, a number of obstacles have to be surpassed. The pluripotency is induced by viral vectors, thus it is generally considered unethical to have them implanted to someone as they host foreign DNA. Another obstacle is being able to turn the pluripotency factors off to allow the

cells to differentiate and stop multiplying. Otherwise the iPSCs will most certainly form a tumour. When the technology is ready, the possibility of forming pluripotent stem cells in a laboratory environment at will, may induce even greater boom of stem cell research. The possibilities and potential of these cells are close to limitless and in some futuristic visions even full organs may be grown in a laboratory from just a handful of iPSCs with patients own genome. (Pittenger, Kerr 2015)

2.3.4 Application of stem cell differentiation

Being able to control stem cell differentiation is imperative in their application in regenerative medicine. Therefore, the issue has evolved into one of the focal points of stem cell research alongside the push for clinical trials (Trounson, Thakar et al. 2011). Nowadays, there is a wide range of information available to guide the differentiation of pluripotent or multipotent stem cells to a desirable direction and to verify the differentiation path the cells have taken. To date, stem cell culture and differentiation serve as an essential model to many human diseases and embryonic development, which holds the keys to the maturation of every adult tissue type.

As stated earlier, pluripotent stem cells, most commonly ESCs, can create any type of cell found in the adult body. While harvesting them has some ethical concerns, ESC is considered the most valuable type of stem cell, valued for its differentiation potency as well as its capability for indefinite self-renewal. Since the first stable human ESC line that was established by Thomson *et al.* (Thomson, Itskovitz-Eldor et al. 1998) the cells have been differentiated *in vitro* to most adult cell lines, a feat that can only be described as daunting. These include neurons (Reubinoff, Itsykson et al. 2001), cardiomyocytes (He, Ma et al. 2003), hepatocytes (Rambhatla, Chiu et al. 2003), pancreatic beta cells (Assady, Maor et al. 2001), endothelial cells (Levenberg, Golub et al. 2002), blood cells (Kaufman, Hanson et al. 2001), chondrocytes (Vats, Bielby et al. 2006) and osteocytes (Bielby, Boccaccini et al. 2004). This proves the pluripotency of ESCs even *in vitro*, but the differentiated cells achieved by these types of studies are still far away from the cells found in functional tissues of an adult individual. Even though the maturing ESCs show markers and function of a differentiated cell type, such as in the case of insulin producing pancreatic beta cells in (Assady, Maor et al. 2001) or in (Zhang, Jiang et al. 2009). While the studies showed a way of producing functional beta cells the production efficiency of the population was only 1-3 % and 25 % respectively. Reported efficiency for differentiation markers can vary much, especially between cell types (Vazin, Freed 2010). This means that only part of the ESCs truly differentiate to adult cell types while most keep their stem cell phenotype. If the population would be implanted in this state, the ESC-like cells could promote tumorigenesis. When combined with the immunoincompatibility of ESCs, the future prospect of using ESCs as a source of implantable cells and tissues is currently bleak at best. Lifelong exhortation to strong immunosuppressant use cannot be a requirement in these types of therapies.

These are the main reasons why iPSCs and ASCs today have more potential for therapeutic use in regenerative medicine. Then again, iPSCs still have the problem of non xeno-free DNA, while ASCs cannot produce all cell types, thus some compromises and significant advancements in techniques are necessary. Either way, cultured iPSCs and ASCs have been shown to express markers for many different mature cells *in vitro*. Many research groups have invented their own protocols for stem cell induction and been able to guide the resultant iPSCs to neural (Vierbuchen, Ostermeier et al. 2010), cardiac (Ieda, Fu et al. 2010), blood cell (Szabo, Rampalli et al. 2010), hepatic (Huang, He et al. 2011), and cartilaginous (Hiramatsu, Sasagawa et al. 2011) differentiation pathways, for example. For ASCs the scale is larger mainly, because of their availability. Especially bone marrow and adipose tissue derived MSCs have a long history of been utilized in cell culture studies for modelling diseases of bone, cartilage and muscle tissues (Ankrum, Karp 2010). One of the advantages of ASCs, or disadvantages, depending on the point of view, is their natural affinity for certain differentiation pathways. This ability makes these cells safer for implantation purposes than their pluripotent counterparts do. Hematopoietic bone marrow transplants have been used as a cure to diseases of blood for some time. The transplanted hematopoietic stem cell population ideally renews the patient's production of blood cells (Hatzimichael, Tuthill 2010). Similar idea is behind the stem cell transplantation to bone (Yamada, Boo et al. 2003), cartilage (Uematsu, Hattori et al. 2005), cardiac muscle (Stamm, Westphal et al. 2003) or neural tissues (Subramanian, Krishnan et al. 2009), for example. These cells are combined with synthetic scaffolds and other tissue engineering concepts to form complete model therapies. A large amount of those are already in clinical trials of phase I and II meaning that they are potentially coming out fast (Trounson, Thakar et al. 2011). On the other hand, in vitro models of stem cell differentiation can be used in medicine development, and to understand disease mechanisms, but these aspects are not generally as prominent from tissue engineering standpoint. These applications hold most of the immediate commercial potential for stem cell differentiation applications, so they should not be underestimated.

Nevertheless, as stated many times before, maturation of stem cells *in vitro* to more differentiated state may be a necessary step to avoid the dangers of implanted stem cells and to shift to the crucial final phases of the clinical trials. Much has been discovered in labs worldwide already, but more basic research for the cues about the differentiation pathways is still needed.

2.4 Concept of cell stretching

When cells are attached to the ECM and other cells in tissues they can feel and react to the mechanical stimuli caused by mechanical stresses. When a ligament is pulled, for example, all the muscle cells in the muscle attached to the ligament can feel the stretch. For this reason, we are able to go to the gym to practice our muscles which in turn start to grow and develop (Gollnick, Armstrong et al. 1973). Nowadays it is known that this

happens when specialized muscle progenitor cells called myosatellite cells sense the increased activity of the muscle, the mechanical stimulus, and begin cell division and differentiation into new muscle fibres (Morgan, Partridge 2003). Similar principle also applies for bone tissue. Our skeleton has evolved to carry the weight of the body against the gravity of the planet. Bone tissue is in normal conditions under constant compressive and torsional stresses, which can be sensed by the osteocytes. As explained earlier in this Chapter, they regulate and maintain the mineralization and the density of the bone tissue (Bonewald, Johnson 2008) and the feedback the cells get from physical stimuli is a key factor on keeping this activity on. Astronauts living in zero gravity, for example, tend to lose bone mass and density during long missions when bone tissue starts to break down due to low amount physical stimuli (Grigoriev, Oganov et al. 1998). This information can be transferred to laboratory setting to subject the cells to controlled mechanical stress to study the effects.

Cells are able to feel the external stimuli through their interaction with their immediate surroundings. All animal cells apart from couple of exceptions require a substrate, a matrix, or another cell to adhere. In tissues, cells are adhered either to the ECM or to each other. Cell-cell junctions can be immobilizing anchors with or without space between cells, channel forming junctions which relay chemical information from cytoplasm to cytoplasm, and signalling junctions such as the nerve cell synapses. Cell-cell junctions enable the formation of concentrated tissues and the flow of information from cell to cell. However, when reacting to an external stimulation, natural or artificial, the cell-ECM interaction holds the most importance. As cells attach to ECM, they form focal adhesion points (Geiger, Spatz et al. 2009). Focal adhesion points are protein complexes outside of the cell that connect actin filaments of the cell cytoskeleton directly to the ECM. They are thus vital in relaying the mechanical stimuli of the surrounding ECM to the cell cytoskeleton inside the cell. This process is called mechanosensing (Luo, Mohan et al. 2013), and the effect it produces in the cell via complex signalling pathways is called mechanotransduction (Wang, Butler et al. 1993).

Mechanosensing and –transduction cause the cellular reaction to the external stimuli, hence their connection is under great interest and focus of many studies today. The underlying mechanisms of the signalling pathways and their outcomes are currently mostly unknown. There are, still, snippets of information available. In osteocytes, polycystin I is essential for their anabolic response to load (Xiao, Dallas et al. 2011), and under shear stress osteocytes are shown to release nitric oxide, adenosine triphosphate, and prostaglandin, a lipid that induces bone formation (Klein-Nulend, van der Plas et al. 1995). Chondrocytes have been shown to react to hydrostatic pressure and compression by releasing cartilage-specific ECM proteins. In turn, a sliding motion on the surface of a 3D scaffold caused increased expression of protein lubricin, which acts as a joint lubricant (Grad, Eglin et al. 2011). Cardiomyocytes in turn have been shown to react to static

stretch by actin filament production and alignment, and by producing branched and striated structures reminiscent of mature sarcomeres. In addition, the mechanical tension kept the contractile proteins, which are vulnerable to enzymatic degradation, protected from being degraded (Simpson, Majeski et al. 1999). It is obvious that cells do actively react to external mechanical stimulation, but how that actually happens remains mostly unproven.

In cell culture setting, mechanotransduction is induced by artificial ECM, ECM proteins or ECM peptides. Nowadays, it is a common practice to prepare the substrate with adhesion proteins in many cell culture applications as it improves cell adhesion and culture viability in many ways. The rule of thumb in general is that the better the cells adhere to the substrate, the better they thrive (Wipff, Majd et al. 2009, Kuddannaya, Chuah et al. 2013). If a growing cell disconnects with the substrate it will in most cases result in apoptosis. Mechanical stimulation of cells during culture is notoriously hard to the cells, and it can be a major inconvenience for engineers who want to evaluate their devices and applications. For example, stretching the substrate can rip any poor or incomplete focal adhesion points (Wipff, Majd et al. 2009) and leave the cell without support. Slow speeds or maximized cell adhesive quality of the substrate minimize the shock caused by stretching.

2.5 Studies and Devices

Nowadays, the cell stretching research utilizes a myriad of applications and devices to apply and measure the stimulation. Because most cell types require some form of a substrate to grow, it must be incorporated into the device design. Whether the cell culture or the substrate is in 2D or 3D, the stretching is applied to the substrate first and then to the cells via focal adhesion points. This means that substrate material requirements for these types of applications have strict limitations. Regardless, researchers have created many imaginative ways to combine stretching systems and cell culture together. Next, various strategies in recent dynamic cell studies are briefly presented. The devices are categorized based on the application principle of the stretch: manual, fluidic, electric motor, or magnetic field controlled CSDs.

Many groups in search of new ways to study cells have noted the good properties of PDMS for cell stretching applications. It is thus of no coincidence that so many cell stretching methods and dynamic culture devices utilize this material. Thin membranes created from PDMS have proven as a great elastic substrate with tuneable properties. Manually applied stretch is likely the simplest method for these devices. In those, the stretch is controlled by the researcher manually by hand and is usually kept static after the initiation. *Wipff et al.* studied various cell adhesive coatings for PDMS cell stretching applications. To test the functionality of the coatings, their effectiveness under mechanical strain, they employed a simple method to apply stretch on the thin PDMS membrane that housed fibroblasts. They used a well on top of the membrane to hold the culture

medium. When the well, that contained cells, medium, and the membrane, was pressed against a ring, which was smaller in diameter that the well, the membrane was stretched. Wipff et al. had mounted the ring on a microscope to see the cells and the stretching process in real time. They also had embedded opaque tracking particles inside the membrane and fluorescent beads on top of the coating to study the transmission of the stretch to the coating and the cells (Wipff, Majd et al. 2009). In similar fashion, *Goffin et al*. had studied the composition of fibroblast focal adhesions under stretch on PDMS membrane with various micropatterns (Goffin, Pittet et al. 2006). Another PDMS membrane based manual stretching device, introduced by *Lee et al.* in 1996 (Lee, Delhaas et al. 1996), was used more recently by *Braakman et al.* to study Schlemm's cells from the eye. The device is able to produce an equiaxial stretch on a PDMS cell culture membrane. The function of the device is as follows: An outer cylinder is attached to a threaded inner cylinder, which in turn is attached to the membrane. The inner walls of the culture well are fixed to press against the membrane. Therefore, when the outer cylinder is rotated, the threads of the inner cylinder enable the downward movement of the outer cylinder, which presses against the fixed walls and the membrane, which is equiaxially stretched up to 20% (Braakman, Pedrigi et al. 2014).

Since the times of the study by *Lee et al*. many systems with different working principles have risen. CSDs controlled by fluidic mechanisms are quite popular and allow for more control over the stretching parameter than manual devices. Air is used to create vacuum pressures while liquids are usually used to create increased pressure upon a flexible membrane. For example, *Zhao et al.* introduced a convenient PDMS membrane based platform for dynamic cell culture. It includes a microfabricated PDMS channel system that is sandwiched by PDMS bulk under it, and a thin membrane on top. When the channel system is filled with air or a liquid, and the pressure in the system is increased, the membrane will deform outwards stretching equiaxially any cells that are grown on top of it. The device implements a very simple and effective mechanism to induce stretching, but the significant vertical movement of the membrane makes it very difficult to monitor the stretching process in real time with a microscope (Zhao, Zhou et al. 2011). This problem was greatly reduced in the similar system published by *Kreutzer et al.*, which is also used in the experimental part of this thesis work. **Error! Reference source not found.**A depicts this device and its working principle. The pneumatic PDMS based CSD includes a circular vacuum chamber around the cell culture well, closed by a glass plate on top and a thin membrane on the bottom. The cells grown on top of the membrane are equiaxially stretched when a vacuum is applied in the chamber as the walls of the inner chamber bend, thus deforming the membrane and chamber causing stretch in the middle in all directions. The vacuum system that can hold dozens of devices at the same time can be attached to a computer that controls the amplitude and frequency of the vacuum pressure enabling long-term cyclic stretching experiments. While some vertical adjustment is required, the well can still be monitored during stretching in real time with a microscope (Kreutzer, Ikonen et al. 2013). A similar pneumatic working principle was utilized by

Huh et al. in their so called lung-on-a-chip device, but its design, as depicted in **Error! Reference source not found.**B, is quite different. It constitutes of two microfabricated channel systems, instead of just one as in the device by *Zhao et al*., separated by only a thin porous PDMS membrane in the middle. On top and below the membrane stands the cell culture chambers flanked by two vacuum chambers. When a negative pressure is applied to the vacuum chambers, it allows the uniaxial stretch of the two cell cultures on the both sides of the membrane. In addition, the pores allow signalling between the two cultures. A microfluidic system on the both sides of the membrane induces shear stress, while providing constant flow of fresh medium to the cells. *Huh et al.* used this innovative co-culture CSD to culture and study epithelial and endothelial cells found in lung alveoli (Huh, Matthews et al. 2010). Very recently, *Ugolini et al*. had the same idea behind the working principle of their CSD. However, they increased the number of chambers on one chip to four allowing easier control of parallel cultures. A microfluidic system for small molecule exchange, similarly to the device by *Huh et al.*, runs under the four cell culture chambers and the membranes. When a negative pressure is applied to the vacuum chambers, the membrane deforms uniaxially. Real time cell monitoring capability and the full cyclic control of the negative pressure completes the system (Ugolini, Rasponi et al. 2016). There are also companies focused on these types of CSDs. Flexcell International Corporation distributes several different stretching systems and related accessories; therefore, their products are quite widely referenced in the field. Also for their systems, the operational basis is a vacuum based stretching of a silicone membrane. The membrane sits on top of a fixed base flanked by vacuum chamber. The vacuum chamber can be circular for equiaxial stretching or rectangular for uniaxial stretching. When the membrane is drawn in the chamber via negative pressure, the middle section is stretched. However, the system needs lubrication under the membrane for it to function properly. A modification of this basic system, called TissueTrain®, can be used as a platform for dynamic long term 3D cell culture (Yang, Rothrauff et al. 2013) or as a set up for dynamic loading for bioartificial tendons (Scott, Danielson et al. 2011), for example.

Motored mechanical CSDs are also popular for their controllability. They can still differ much in design from one another, but in general, the working principle is the same: a motor induces physical movement that is transferred to a flexible cell culture substrate either straight or via lever based transmission. The simplest example, the pulling clamp, is a popular operational mechanism for these types of CSDs. In these, the flexible substrate, which is often PDMS, is physically clamped to a lever leading to rotating or linear motor that pulls on the substrate, creating uniaxial stretch. *Ahmed et al*. used this type of device to study a hydrogel coated, micropatterned and myoblast seeded PDMS well under cyclic stretch (Ahmed, Wolfram et al. 2010). *Greiner et al*. did the same, except with dermal fibroblasts (Greiner, Hoffmann et al. 2014). Devices with this mechanism fit well for researchers undergoing a prototyping phase, because the design can hold any devices, wells or substrates that fit to the clamp, which can likewise be modified. *Figueroa et al*. used a very similar setup to study endothelial cell responses (Figueroa, Kemeny et al.

2011), as did *Leong et al*. when culturing MSCs under cyclic stretch, but on polycaprolactone substrate instead of silicone (Leong, Wu et al. 2012). Alongside their other study, *Ahmed et al*. introduced a different pulling clamp device that used linear actuator for displacement. The stretch with the device can reach up to 45 % while allowing real time observation with a microscope (Ahmed, Kural et al. 2010). *Li et al.* proposed a different approach by experimenting with a new stretchable, biocompatible and striated fugitive glue based substrate. The material can withstand at least 700 % stretch and according to *Li et al.*, the material could be used in many applications in place of the more commonly used silicones (Li, Lucioni et al. 2015).

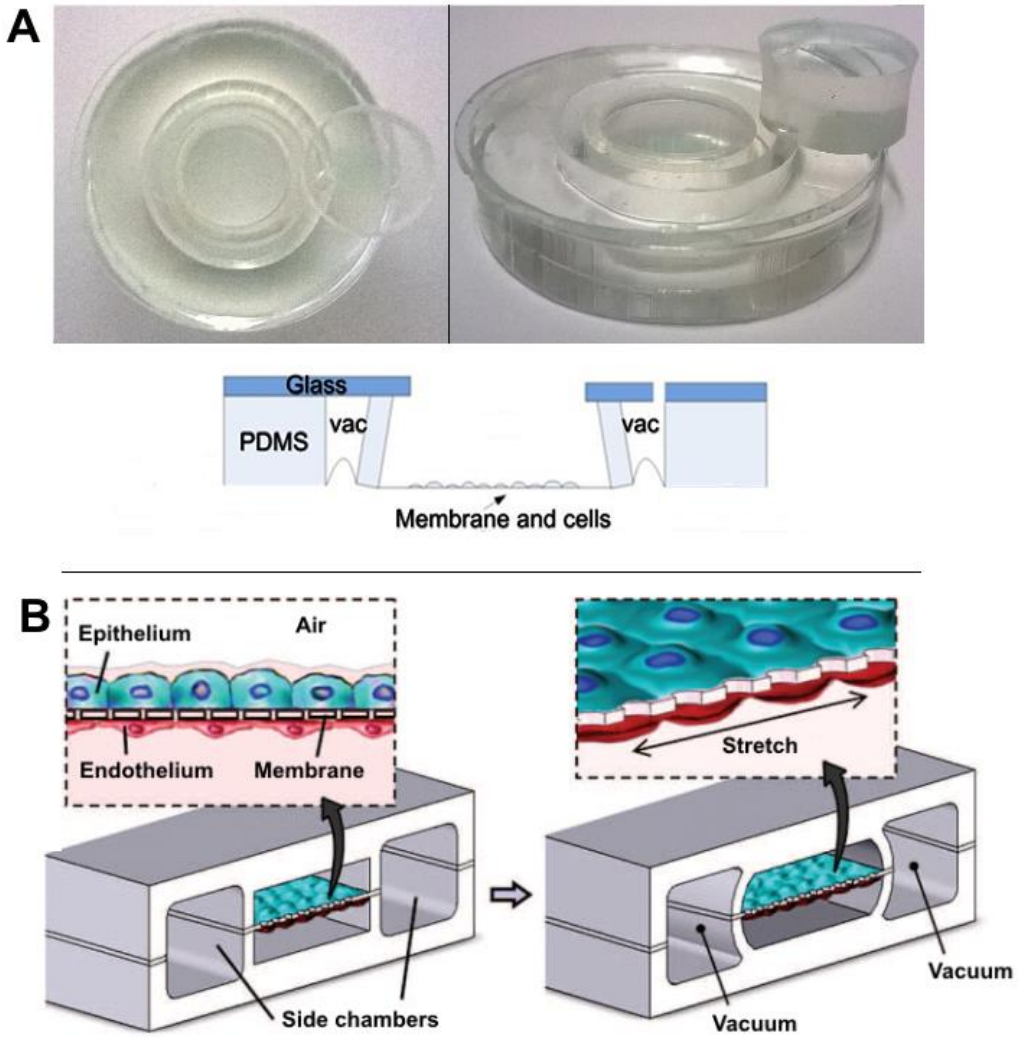

Figure 2. *The pneumatic CSDs and their working principles as described by (A) Kreutzer et al. and (B) Huh et al. (Huh, Matthews et al. 2010, Kreutzer, Ikonen et al. 2013).*

A more complex device than pulling clamps, the IsoStretcher is a recent addition to the ever-growing number of complete cell stretching systems. Published by *Schürmann et al*., the IsoStretcher is a complicated mechanical device for expanding the PDMS based cell culture membrane. Figure 3A shows the location of the membrane in relation to the

other parts of the device. The device is driven via a stepper motor that rotates the lowest ring that houses six pins used to transfer the displacement to the membrane. The pins follow a tangential trajectory as the lowest ring rotates pulling the membrane with holes for the pins outwards, creating a constant equiaxial stretch to the membrane. According to *Schürmann et al*., the stretch capability of the device goes up to 20 percent (Schurmann, Wagner et al. 2016). The cellerator, as the device is named by some sources, by Cytomec GmbH has been the device of choice by several studies of late. This iris-like motored mechanical PDMS membrane based CSD has been used to study MSCs (Majd, Quinn et al. 2011), chondrocytes (Rosenzweig, Matmati et al. 2012, Rosenzweig, Chicatun et al. 2013) and myofibroblasts (Klingberg, Chow et al. 2014). In this device, the membrane that holds the cells is attached to the walls of the culture well, which include holes for the 'arms' of the iris-like mechanism. As the arms, which are attached to the outer edge of the device, are displaced outwards by an external motor, the membrane is equiaxially stretched. This displacement mechanism is illustrated in Figure 3B. *Rosenzweig et al.* report a staggering 600 % increase in surface area after the chondrocyte culture had been expanded continuously for 13 days (Rosenzweig, Matmati et al. 2012). The maximum stretch capacity for the device is reportedly 800 % which is by far the highest of the devices presented here (Rosenzweig, Chicatun et al. 2013).

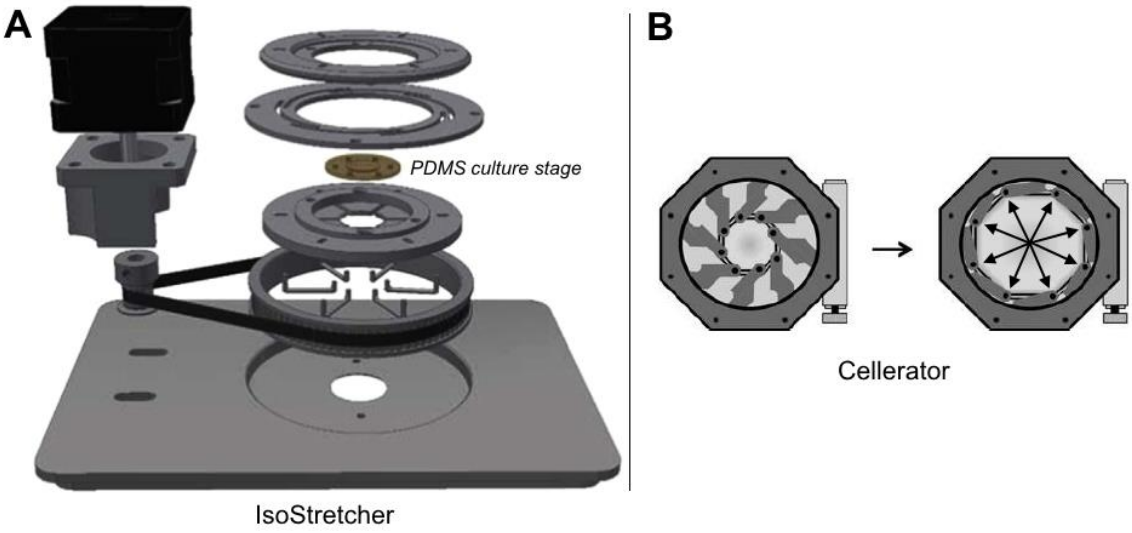

Figure 3. *(A) Deconstruction of the IsoStretcher CSD with highlighted PDMS based cell culture area (Schurmann, Wagner et al. 2016). (B) Illustration of the iris-like mechanism of the cellerator CSD (Rosenzweig, Matmati et al. 2012).*

By utilizing electromagnetic principles, *Mayer et al*. had a very different approach in their device, when compared to the others introduced in this Section. They embedded carbonyl iron particles into 2 mm thick ultra-soft PDMS bulk. The premise was to be able to deform the created magnetoactive elastomer, as they call it, with external magnetic field. In their study, the magnetoactive PDMS piece sits inside a petri dish on top of either permanent magnet or controlled electromagnet based yoke. The former produces static magnetic

field and strain, while the latter system can create changing magnetic flux, thus changing strain on the magnetoactive substrate. *Mayer et al.* also introduced a 24-well based stretching platform for simultaneous stretching on half of the wells (Mayer, Rabindranath et al. 2013).

The list could go on almost indefinitely, which only shows how imaginative researchers and engineers are on this field. However, to reach the next milestone in tissue engineering and dynamic cell culture applications, more complex tissue types and stem cells need to be analysed in different dynamic environments to get the crucial information about the cues that lead to healthy tissues *in vitro*. Now, the research is usually focused on the functionality of the devices, rather than the cell culture. The fact is that the field is still a Wild West of different protocols and devices where it is often difficult to project the results of external studies to one's own. Standardization and collaboration in every phase, especially in the cell culture phase, is gravely needed or the process to human spare parts will continue being needlessly cumbersome.

3. POLYDIMETHYLSILOXANE SURFACE MODIFICATION

PDMS must be one of the most widely used organosilicon materials to date. It sees use today as a lubricant, sealant, food and shampoo additive, toy, resistive coating, contact lenses, and various medical devices to name some. PDMS elastomers are especially useful in medical grade device research and development. PDMS in its commercially available elastomer composite form is inert to most chemical reactions, easy to handle and shape, is not a source of cytotoxicity, and when needed can be activated for a wide range of chemical reactions.

This Chapter focuses on the material and chemical characteristics of PDMS. First, relevant chemical and physical characteristics of PDMS are explained thoroughly. Then, basic surface treatment methods or techniques are presented based on the nature of the treatment that can be either plasma, physical, or chemical. Finally, some of the most relevant advanced surface treatment methods used in studies in biomedical field are explored and described.

3.1 Chemical characteristics of PDMS

As its name suggests, PDMS is an organosilicon compound with a siloxane backbone. It is a specific polysiloxane with two methyl groups attached to each silicon in the siloxane Si-O-Si backbone (Figure 4). The polar siloxane oxygen atoms in the backbone and the non-polar methyl groups make up most of the chemical nature of PDMS. The most notable characteristic of PDMS is probably its high hydrophobicity, which results directly from the methyls flanking the siloxane backbone. The flexibility of the polymer chain also permits the arrangement of the methyls on the surface of the polymer bulk regardless of it being in elastomer or liquid form. The chain flexibility arises from the large bond angle of the siloxane, and it enables the polymer in liquid form to spread out on surfaces and copy its features up to nanoscale. Therefore, elastomeric PDMS is widely used in soft lithography to produce moulds or stamps with very high resolution (Rolland, Hagberg et al. 2004). The glass transitional temperature of PDMS reflects this mobility and is generally under -120 °C, while the intrinsic hydrophobicity gives PDMS one of the lowest surface energies known. It can be simultaneously a curse and a blessing for its usability in the medical field. Table 1 lists some of the most referenced characteristics of PDMS. (Poojari 2014)

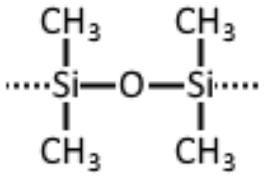

Figure 4. *PDMS polymer chain subunit: Si-O-Si = siloxane, CH_3 = methyl.*

Table 1. *Qualitative PDMS characteristics edited from (Rodrigues, Dourado 2014).*

Biological	***Physicochemical***
Non-toxic / inertness	Thermal stability
Moderate biocompatibility	Resistance to UV, oxygen, ozone, sunlight
Protein adsorption / absorption	Resistance to aging and biodegradation
Hemocompatibility	Sterilisable and low curing temperature
Poor microbial adhesion	Excellent dielectric behaviour
	Low density, high molecular weight
	Chemical stability, resistance to hydrocarbons, oils and solvents
	Flexibility / elasticity, easy to mould and shape
	High gas permeability
	Good optical transparency
	Hydrophobic character

3.2 Elastomeric PDMS composite Sylgard® 184

As suggested, PDMS is found in many conformations, but its elastomer composite is the most important one from the point of view of biomedical technologies. Probably the most widely referenced brands of PDMS composites in this field are distributed by Dow Corning. Because linear PDMS polymer chains are so flexible that they are naturally in liquid form, they have to be crosslinked to solidify them. The amount of crosslinking reactions ultimately decides the hardness or elasticity of the PDMS composite in solid-phase. Controlling this reaction is easy with well-known and well-established PDMS elastomer kits, which are widely available for purchase, and have standardized crosslinkable side groups added to the basic backbone of PDMS. The side groups are generally activated through thermally active catalyst, which crosslinks the polymer chains permanently through covalent bonds.

As mentioned above, Sylgard® 184 silicone elastomer from Dow Corning is one of the most referenced PDMS composites in scientific studies, and for clarity, in this thesis PDMS refers specifically to Sylgard® 184 composite from now on. It is a two part (base and curing agent) heat curable polymer with a platinum-based catalyst and ethylbenzene as a solvent (Dow Corning 2015, Dow Corning 2016). The base polymer solution consists

of long ~60 subunit PDMS chains terminated by vinyl (=CH_2) groups while the curing agent polymer solution contains, in addition to the long chains, shorter 9-13 subunit polymer chains which have at least three of the methyl side groups replaced by hydride (-H) groups (Lisensky, Campbell et al. 1999). These hydride groups act as the points for the crosslinking reaction between the long and short polymer chains. The platinum catalyst causes an addition reaction of the hydride to the carbon-carbon double bond of the terminal vinyl group (Speier, Webster et al. 1957) that is accelerated by temperature (Figure 5). The result is an irreversible network of long flexible PDMS chains that begin and end to the sides of the shorter chains. These crosslinking points allow the elastic recovery of the material after strain. When a strain is applied to the material, the low energy backbone chains slide past each other allowing deformation. When the strain is in turn released, the crosslinking points force the material to its initial shape. The Young's modulus i.e. the stiffness of PDMS composites can be controlled by varying the ratio of curing agent polymer solution to the base polymer solution. As the crosslinking increases in the bulk of the polymer, so does the stiffness. Nonetheless, PDMS retains most of its flexibility caused by the siloxane backbone, and the freely rotating methyls, even after curing. Therefore, the surface is generally in constant motion when the longer chains twist and allow the shorter uncrosslinked chains to seep through to the surface.

It is a well-known fact that PDMS exhibits hydrophobic recovery after modification of the surface with hydrophilic groups (Bodas, Khan-Malek 2007). It has also been shown that removing those short free chains from PDMS can inhibit this recovery (Eddington, Puccinelli et al. 2006). Storing hydrophilized PDMS in water also inhibits the recovery, as does storing it in liquid nitrogen (Everaert, Van et al. 1996). This behaviour can easily be explained by understanding what affects the chain mobility in the PDMS bulk. The water creates a polar environment at the PDMS-water interface, which anchors the hydrophilic group containing chains to the surface due to hydrogen bonding, thus inhibiting movement of hydrophobic chains from the bulk to the surface. For a similar reason storing hydrophilized PDMS in air creates a non-polar PDMS-air interface, which attracts chains containing hydrophobic methyl side groups , thus leading to hydrophobic recovery. Storing the modified PDMS in liquid nitrogen, on the other hand, lowers the temperature so low that the chain mobility, and the hydrophobic recovery, is reduced to a minimum (Everaert, Van et al. 1996). Additionally, it could be hypothesized that even a hydrophilic recovery is possible after hydrophobic recovery, if the PDMS is stored in polar solvent such as water.

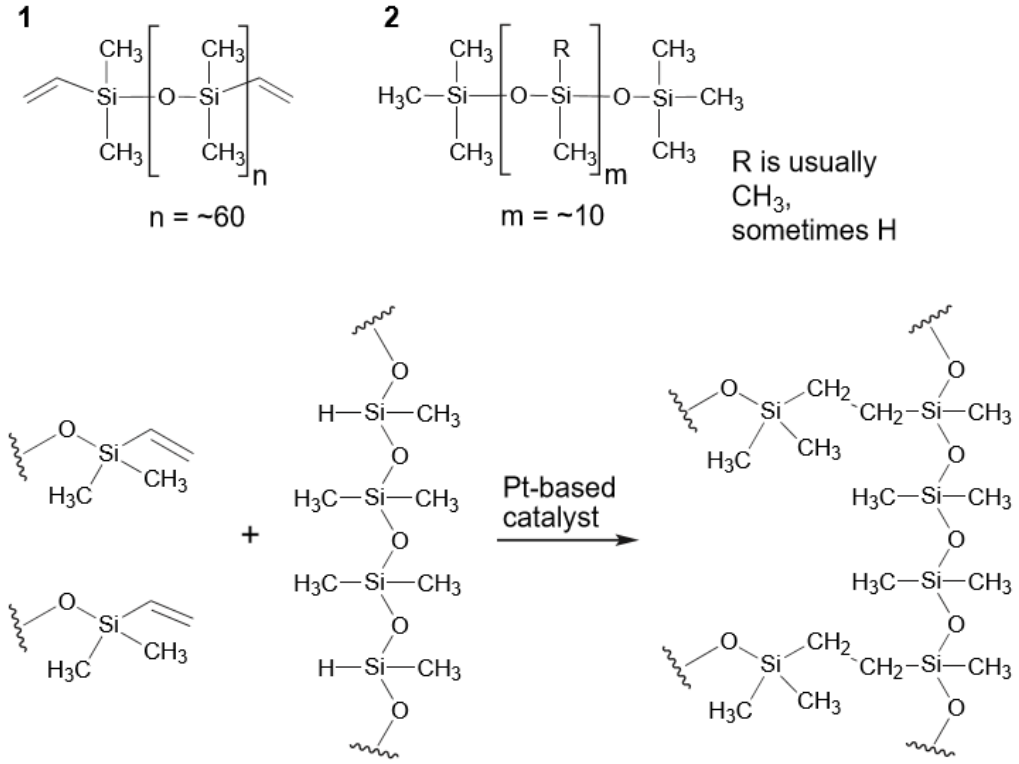

Figure 5. *Platinum catalysed Sylgard® 184 curing reaction between the base (1) polymer and curing agent (2) polymer chains (Lisensky, Campbell et al. 1999).*

The hydrophobic nature of pristine PDMS surface can easily be verified by water contact angle measurement, and it is usually around 110° (Mark 2009). For this reason, and the chain mobility, PDMS has a tendency of adsorbing any hydrophobic molecules it meets. Many proteins, for example, can passively bind to PDMS surface as the weak van der Waals' forces immobilize them. The immobilization by electrostatic force caused by a very weak negative charge is also possible. Sylgard® 184's electric charge is somewhat anomalous in nature, as pure PDMS is electrically neutral. However, there is some evidence that suggests it is caused by additive materials added by the polymer manufacturer (Ocvirk, Munroe et al. 2000). The hydrophobicity is not present only on the surface, so hydrophobic proteins wandering to the surface may be partly absorbed inside the PDMS. The chain mobility and the nature of the crosslinked polymer network make PDMS highly permeable to gases and small molecules including some proteins. It can cause fouling of the polymer surface as the hydrophobic parts of the proteins become trapped (Toepke, Beebe 2006). Furthermore, non-polar solvents readily diffuse into the PDMS bulk and can cause substantial swelling and deformation (Dangla, Gallaire et al. 2010). If the absorbed solvent is allowed to evaporate, PDMS will return to its original dimensions as the solvent escapes the bulk in gas form. All of this shows that understanding the chemical structure and chain mobility can lead to understanding the way PDMS functions in different situations and help predict its behaviour in novel applications. (Huszank 2014, Rodrigues, Dourado 2014)

3.3 Surface treatment of PDMS

As mentioned many times already in this thesis and elsewhere, PDMS elastomer's surface is unsuitable for cell culture in its pristine state. Its hydrophobicity is the main culprit as cells have been shown to favour more hydrophilic substrates, or to be exact, substrates with higher surface energy density (Zhao, Raines et al. 2007). Most surface treatments of PDMS aim to increase the surface energy density to make the substrate more accessible to water molecules and chemical reactions. In short, pristine PDMS surface does not offer any attachment opportunities for the cells, which usually leads to quick cell death and culture failure. While pristine PDMS is prone to adsorbing proteins, as stated previously, it is not efficient enough to create sufficient substrate for all cell culture needs. To culture cells on PDMS, these problems must be circumvented.

When the cell culture is dynamic, the requirements for a good substrate grow exponentially. Additionally, these dynamic systems are usually designed for studying human stem cells which tend to be more sensitive to the culture conditions, including the substrate, than animal or immortal cancer cell lines (Rosler, Fisk et al. 2004). Luckily, there are many possibilities to approach this issue. As the knowledge and technology has gone forward, more methods for surface treatment have also been created and studied. Still, a major part of surface treatment methods used for cell culture studies have relied on the few well established and simple methods of plasma oxidation or physisorption (Wipff, Majd et al. 2009, Kreutzer, Ikonen et al. 2013). On the other hand, optimizing novel coating methods is usually so laborious and complicated that cell culture researchers and tissue engineers would rather focus on taking an existing method without the need to focus on the chemistry of the treatments and optimization steps. Regardless, there are many methods that can vary greatly in their utilization. In general, they can be classified into physical and chemical methods depending on what type of interaction dominates during the surface treatment. The division between the methods can be seen in Figure 6. This classification is not fixed as some treatments could be labelled as both, or a mix of both, as it will be explained in the following Sections. (Qiu, Wu et al. 2014)

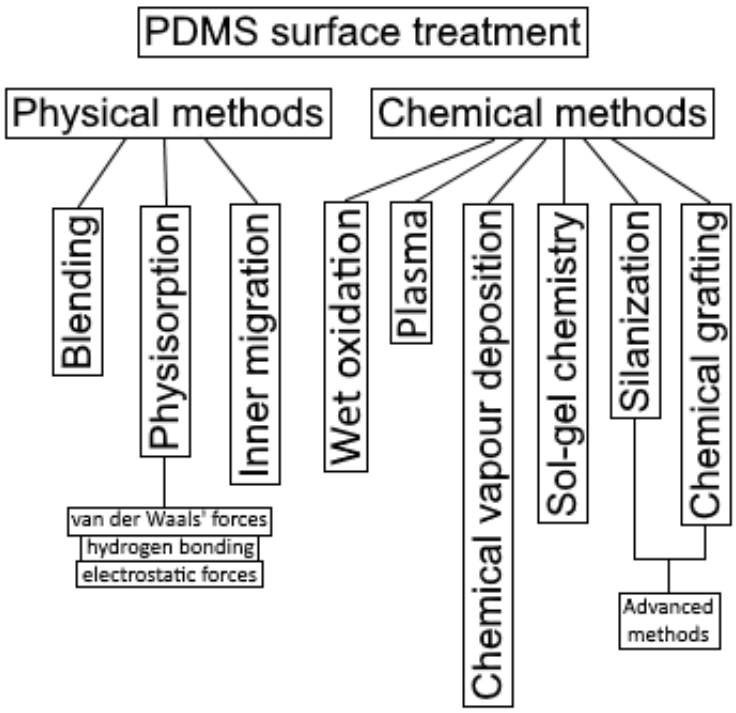

Figure 6. *A list of PDMS surface treatment methods divided by their classification into physical and chemical methods.*

3.3.1 Plasma treatments

Plasma treatment of PDMS, which technically falls under chemical methods, is one of the simplest and most used surface modification methods. It is used to counteract the intrinsic hydrophobicity of the material, effectively turning its surface chemistry upside down. Plasma is a gas in ionized form, sometimes called the fourth fundamental state of matter, where all molecular bonds between atoms have been dissociated. It is usually created from a gas by heating or strong electromagnetic field. The plasma is filled with charged free radicals that are highly reactive even with low surface energy materials such as PDMS. The most commonly used plasma gas sources for PDMS include oxygen, nitrogen, argon, ammonia, water and air. All of these focus on the modification of the surface chemistry and cause the weak hydrides (-H) on siloxane backbone to be replaced mainly by hydroxyl (-OH) or amine ($-NH_2$) groups. The hydrogens on the methyl side group and the methyls themselves are also susceptible to addition reactions by the radicals (Yang, Yuan 2016). Oxygen plasma creates hydroxyls, while nitrogen and ammonia mainly produce amines. Oxygen containing water and air plasmas also mainly introduce oxygen species to the surface, which indicates higher reactivity for oxygen radicals. For example, argon plasma does not introduce anything to the surface by itself, but the oxygen leftovers from air and moisture are enough to introduce oxygen species to the surface (Pinto, Alves et al. 2010). In non-ultra-vacuum plasma systems, this can cause problems, when the oxygen traces disturb nitrogen addition to the PDMS surface, for example. The two most common applications of plasma on PDMS are increasing the hydrophilicity and

adhesion. PDMS can be covalently bonded with itself or glass after oxygen plasma treatment due to silanol-silanol (Si-OH) reaction, which produces a siloxane (Si-O-Si) bond and releases water. Similarly, nitrogen plasma treated surface with amines is capable of binding epoxy groups (Yang, Yuan 2016). Epoxy groups can be found on the surface of SU-8, for example, which is a photoresist material commonly used in fabrication of microfluidic devices. While they cannot be classified as plasma, ozone treatment and ultraviolet radiation have also been used for similar effect as oxygen plasma. They tend to be much slower, but can reach deeper into the PDMS bulk. (Qiu, Wu et al. 2014)

3.3.2 Physical methods

The main characteristic for physical surface treatment methods is the type of interaction between PDMS and the molecule used for the treatment. It is physical in nature, meaning no chemical reaction happens between the molecule and PDMS. The most relevant physical methods can be further classified into blending, inner migration and physisorption.

Blending is one of the least used methods for PDMS surface treatment. Nevertheless, when PDMS is in liquid phase, other polymers and materials can be blended in the bulk. The molecules are spread out and immobilized inside the bulk after curing, while having an effect also on the surface properties (Xiao, Yu et al. 2007). After blending, if the blended molecules are small enough and have certain properties, it is possible to exploit the relatively mobile bulk of PDMS to passively direct the blended molecules to the surface. This method is called inner migration. Amphiphilic molecules, such as many of the polymers in the pluronic family, can be belended with PDMS, and when it is placed in water, the amphiphilic molecules begin to diffuse via gradient-induced migration. The hydrophilic parts of the amphiphilic molecules migrate to the surface and orient themselves towards the water, which leads to hydrophilization of the PDMS surface that can last for months (Wu, Hjort 2009).

Physisorption, or physical adsorption, utilizes the physical interaction between the external molecules and the PDMS molecules on the surface. The physical interaction can be van der Waals' force, hydrogen bonding or electrostatic force depending on the paired molecule or the state of the PDMS surface. By using a melt or a solution, the molecules are brought into physical contact with the PDMS surface. If the PDMS is pristine, hydrophobic interactions dominate, and molecules can be adsorbed to the surface from their hydrophobic functional groups. Amphiphilic molecules such as surfactants and large or hydrophobic proteins can have a strong interaction with the PDMS surface. If the interaction is stronger than with the solution, they will stay on the surface after the solvent has been removed. If the PDMS has been treated with oxygen plasma to have polar hydroxyls on the surface, hydrophilic interactions, in other words hydrogen bonding, will dominate. Molecules with functional groups capable of hydrogen bonding can then bind to the PDMS surface. However, because water will also bind to these points, the binding affinity of the molecules could be lower in aqueous solutions.

In electrostatic interaction, the negatively and positively charged functional groups in molecules attract each other and potentially cause adsorption. Pristine PDMS of some manufacturers, such as Dow Corning, is reportedly slightly negatively charged (Ocvirk, Munroe et al. 2000) and capable of binding molecules with positively charged functional groups. This can be useful for some big proteins which can have several different functional groups, some with positive charge and some with hydrophobic inert groups. Treating the PDMS with plasma that introduces pH sensitive groups (acidic -OH, basic $–NH_2$), for example, can be useful to introduce either positive or negative charge to the surface in a controlled way. Acidic groups have a leaving H^+ ion that give a negatively charged surface, while basic groups accept the H^+ ion giving a positively charged surface. Electrostatic interaction is also exploited in the more advanced layer-by-layer (LBL) method that is revisited later in this Chapter. Physical methods described here have shown promise by being easy to implement and cheap, but in cell culture applications and most notably dynamic cell culture applications they tend to fall short by not being powerful enough. The more advanced methods that focus on chemical bonding instead of physical interactions tend to be more popular in those applications. (Qiu, Wu et al. 2014)

3.3.3 Chemical methods

In chemical surface treatment methods, a chemical reaction happens between the PDMS surface and a molecule that is wanted on the surface. This causes changes in the covalent bonds by either introducing new bonds and/or breaking old bonds. Here, the most relevant chemical methods are divided into wet oxidation, chemical vapour deposition (CVD), sol-gel chemistry, silanization and chemical grafting.

Wet oxidation is a special treatment method that is commonly used for the hydrophilization of PDMS surface in place of a plasma treatment. By washing the PDMS surface with the extremely reactive piranha solution, which is composed of sulphuric acid and hydrogen peroxide, the surface methyl groups are replaced by hydroxyl groups. The result is a nearly complete hydrophilization of the surface.

CVD is a chemical process that is used to produce thin films or coatings from volatile compounds. The substrate is exposed to the gas or vapour, which then reacts with the surface potentially causing a change in the chemistry on the surface. One of the main advantages of CVD treatment is that it is solvent free and can be completely dry. Many of the most relevant CVD treatments for PDMS rely on the vaporization of monomers that spontaneously polymerize on the PDMS creating a surface that can be tailored for the needs of the application (Chen, McClelland et al. 2008). While usually being chemical method, CVD polymer film does not necessarily bind covalently to pristine PDMS, but rather by physical interaction. For covalent CVD, it may be necessary to pre-treat PDMS with plasma, or other activating treatment (Bhushan, Hansford et al. 2006).

Sol-gel chemistry, or method, is a wet process that creates solid material from the monomers dissolved in an aqueous solution: Sol stands for colloidal solution, and gel the formed solid network. Similarly to CVD, the composition of the final coating can be tailored to the needs of the application via the used monomers. Both polymer plastic (Suzuki, Yamada et al. 2010) or metal oxide (Roman, Culbertson 2006) coatings can be created using this method. The advantage of this method is that it creates a homogenously distributed coating. The density of the coating can be controlled, as the remaining liquid is evaporated (Roman, Culbertson 2006). Biomolecules such as enzymes, proteins, or whole cells can also be incorporated into the coating during its formation to create fully biocompatible or bioactive coatings (Böttcher 2000).

The next method, silanization, is a widely used coating method for PDMS that utilizes a class of molecules called silanes. Alkoxysilanes contain one to three alkoxy functional groups (usually methoxy or ethoxy) around a silicon atom, while halosilanes contain one to three halides (usually chloride, fluoride or mix of both). Additionally, at least one another functional group is situated at the other end of the molecule; usually at the end of a short carbon chain. Hydroxyl groups, which can easily be created on the PDMS surface, can readily replace alkoxy or halide groups forming strong siloxane (Si-O-Si) bonds between PDMS and the silane. The clear advantage of this surface treatment method is its capability to introduce a wide range of functional groups covalently to the PDMS surface. Amine (Wipff, Majd et al. 2009, Séguin, McLachlan et al. 2010), epoxide (Moorcroft, Meuleman et al. 2005) and thiol (Séguin, McLachlan et al. 2010) are common functional groups in silanization treatments. During the silanization process, the silane self-assembles into a monolayer. The carbon chain with the functional group points outwards from the surface causing the surface to adopt the chemical properties of that functional group. Groups such as amine and epoxide can be further utilized in binding reactions, for example.

Chemical grafting is a broad term for chemically attaching polymers to a substrate; silanization is basically chemical grafting that is focused on silanes. Chemical grafting can be divided into “grafting to” and “grafting from” techniques, both of which focus on attaching polymers covalently to the substrate. In this case it is PDMS, so generally an activation step, such as plasma treatment, is needed before anything can be attached to the surface. “Grafting to” refers to attaching complete polymers to the surface. It usually leads to rough and irregular surface, especially with long polymers, as they may form clumps and kinks near the surface, as shown in Figure 7. However, “grafting to” is the more straightforward method for creating a chemically tailored surface. Because the polymers are created and functionalized beforehand, there is no need to control the reactions except the grafting itself. “Grafting from” refers to attaching monomers or oligomers to the surface, which act as the starting point for a polymerization reaction. The polymerization continues until the reaction is chemically stopped, or when the monomers run out. This is advantageous, because the polymers grow from the surface in a controlled fashion.

They grow outwards and tend to form dense tightly packed coatings (Hemmilä, Cauich-Rodríguez et al. 2012) as visualized in Figure 7. The basic chemical methods presented here often act as the starting points to the more specific and advanced surface treatment methods required by cell stretching applications, for example. (Qiu, Wu et al. 2014)

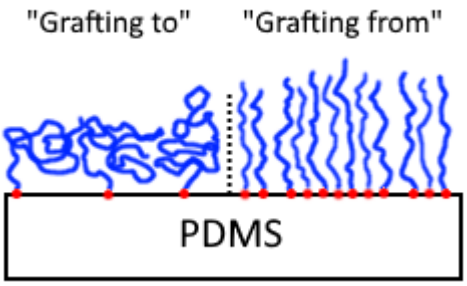

Figure 7. *Visualization of the tertiary structure of "grafting to" and "grafting from" polymer coatings on PDMS.*

3.3.4 Basis for advanced surface treatment of PDMS

When developing novel surface treatment methods, or when replicating already proven methods, it is useful to realize the forces in work during the treatments. More often than not, a chemical reaction between two functional groups of two different molecules dictates the result of the treatment. While nowadays it is possible to buy many types of coating kits aimed at researchers, who are not experts in the field of surface treatment, the lack of knowledge about the surface chemistry can lead to hard-to-decipher results in biomedical applications. The chemistries between PDMS and the reactant molecules, between PDMS and various proteins, or between reactant molecules and cells can quickly accumulate variables, which in face of problems or unexpected results can completely ruin experiments. Factors such as temperature, pH, solvent, time, surface impurities, surface state after plasma treatment etc. can have a critical effect on the outcome of a binding reaction. For a chemist, such factors should not be alien, but for cell biologists, and biomedical engineers focusing on research in cell culture or biomedical implant and device applications, the knowledge base about such factors is not as pronounced. Broad multidisciplinary approach is critical within complex topics, such as cell stretching applications, where expertise in cell culture, microfabrication, polymer science, chemistry, biomedical engineering, and tissue engineering have to meet in order to truly advance to the next level of research. Tissues and organs build from ground up in a laboratory might be far away now, but with the strong institution based multidisciplinary research mankind is closer to the goal than ever before.

As stated many times earlier in this Chapter, pristine PDMS surface is unsuitable for cell culture, and many other applications. However, as an elastomer material with other beneficial properties, PDMS is too advantageous to be discarded. This has led to the many different surface treatment methods aimed at overcoming the material's shortcomings of

low reactivity, involuntary molecule adsorption, and hydrophobicity, as explained before in this Chapter. However, these methods are very basic and not always ideal for many applications. Therefore, some methods have been taken further by introducing multistep treatments, immobilizations, layer-by-layer methods etc. The next section introduces various advanced surface treatment methods for PDMS that have been used in biomedical research.

3.3.5 Advanced surface treatment of PDMS in biomedical research

Two generalizations can be made about the advanced surface treatment methods for PDMS in biomedical research; 1) the focus is in layer-by-layer or step-by-step techniques where multiple treatments are made into one method; 2) biocompatibility is the main concern in PDMS based applications and some form of protein immobilization is usually used to solve this issue. In biomedical field, PDMS is used as a base material in cell culture, biomedical implant, microfluidics, or biosensor applications. For cell culture applications, whether they are dynamic or static, it is necessary to achieve some form of cell adhesive surface. The most popular strategy is to bind ECM components covalently to the surface to let the cells adhere there naturally. Plasma treatment is also a popular choice to activate the inert PDMS surface for further binding reactions, as mentioned many times before.

Séguin *et al.* used a multistep process to coat their PDMS based microfluidic assay. A thin membrane of PDMS was covered by a steel mesh with circular micropatterns before exposing the surface to argon plasma and sputtered aluminium. After the etching step, the PDMS surface contained active and inactive sections, because of the steel mesh used for masking parts of the surface. The surface was silanized with amine terminated silane and thiol terminated silane. Amine reactive bissulfosuccinimidyl suberate was then used to crosslink and immobilize protein-A covalently to the surface to act as a catcher for certain immunoglobulins. The reaction is possible via bissulfosuccinimidyl suberate molecule's n-hydroxysuccinimide (NHS) esters on both ends. Sodium salt of this can be seen in Figure 8. The NHS acts as a leaving group in the reaction with the amine, forming a strong amide bond. The method Séguin *et al.* proposed is a powerful way to inhibit the surface treatment only to desired parts and showed that cells can be guided to these areas. (Séguin, McLachlan et al. 2010)

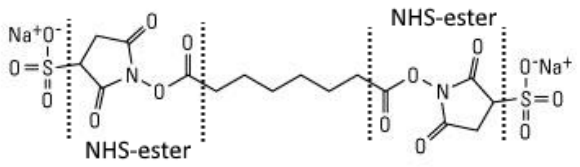

Figure 8. *Highly soluble sodium salt of bissulfosuccinimidyl suberate edited from (Pierce Biotechnology 2012).*

Wipff *et al.* focused on improving the amount of bound collagen type I on their PDMS based CSD. After activating the PDMS surface with oxygen plasma, they silanized the PDMS surface with (3-aminopropyl)triethoxysilane (APTES) that creates free amines on the surface. To bind collagen they used a glutaraldehyde (GA) based crosslinking. GA is a short dialdehyde molecule with aldehyde groups at the both ends of a five carbon chain. Aldehyde is reactive towards amines with its carbonyl group and this reaction creates an imine bond under acidic environment. The imine bond is a covalent linkage, but it is susceptible to spontaneous hydrolysis, which can be a problem, if the immobilized molecule (e.g. protein) is held by only one imine bond. Collagen molecules, though, have multiple amino acids which contain amines that can act as points for crosslinking by amine reactive molecules. In the study, Wipff *et al*. used collagen type I to functionalize the PDMS devices with cell adhesive properties. They showed that covalently immobilized collagen coating was able to withstand stretching better than physisorbed or electrostatic LBL coating after 48 hours of culture. Fewer detachments of focal adhesion points were detected in covalently modified samples. The electrostatic LBL coating method they also tested included six alternating layers of positively charged polyethylene imine and negatively charged polystyrene sulfonate on oxygen plasma treated PDMS. Therefore, collagen was lying on a negatively charged layer, thus held by electrostatic forces; collagen fibrils are reportedly positively charged (Hadley, Meek et al. 1998). Even though LBL method used seven layers between PDMS and the cells, Wipff et al. showed that stretch applied fully from PDMS onto the surface of the coating. (Wipff, Majd et al. 2009)

Nishikawa *et al.* brought up another covalent collagen immobilization method for cell culture applications. After a plasma treatment and aminosilanization, they used sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)ethyl-3-dithiopropionate (sulfo-SAND) to covalently bind collagen to the surface. Sulfo-SAND is a bifunctional molecule with nitrophenyl azide on the other end, where the azide (-N=N=N) acts as the reactive species, and sulfo-NHS-ester on the other end. The nitrophenyl azide is a photoreactive functional group that can react with a wide array of nucleophilic groups, including primary amines (Clayden, Greeves et al. 2001). As Nishikawa *et al.* show, after the activation by ultraviolet light, the azide releases nitrogen gas and reacts with the amine functionalized PDMS. The NHS side of the molecule points outwards and reacts with the amines of the added collagen, immobilizing it to the surface. Hepatocytes could successfully be cultured on PDMS treated via this method. (Nishikawa, Yamamoto et al. 2008)

Salber *et al.* as well had the idea that PDMS is well suited for their dynamic cell culture application, but to be successful at that PDMS needs to be properly coated. They used ammonia plasma to introduce amines straight onto PDMS surface without the need of a silanization or equivalent step. As a crosslinker for cell adhesion peptides, they used the so called star polyethylene glycol (PEG). In star PEG, six PEG chains branch from a central core group creating its star like appearance. The branches end into isocyanate

functional groups (-N=C=O), which react towards amines, and crosslink the amine functionalized PDMS with cell adhesive molecules. In their study, Salber *et al.* bound synthetic peptide sequences known to interact with cell-binding sites derived from fibronectin, laminin and collagen type IV. (Salber, Gräter et al. 2007)

Related to the previously mentioned study by Salber *et al.*, Ahmed *et al.* used a similar approach to coating stretchable PDMS substrates. After using ammonia plasma, they treated the substrate with star shaped PEG-polypropylene glycol copolymer. The isocyanate groups were used to covalently bind fibronectin applied by stripe patterned PDMS stamp, a method commonly referred to as micro contact printing. The mouse skeletal myoblasts were successfully restricted to areas with fibronectin, and adhered cells were able to survive the four days of dynamic culture. (Ahmed, Wolfram et al. 2010)

Trappmann *et al.* studied human epidermal stem cell differentiation on PDMS functionalized with collagen type I. Without the use of plasma functionalization step, nevertheless, they were able to covalently crosslink collagen onto PDMS by using sulfosuccinimidyl-6-(4-azido-2-nitrophenylamino)hexanoate (sulfo-SANPAH). Sulfo-SANPAH is chemically related to sulfo-SAND, and thus reacts under the same conditions in similar manner. However, Trappmann *et al.* induced 365 nm UV-irradiation, as the solution containing the crosslinker was in contact with pristine PDMS. Normally, no reaction would occur, but the UV-irradiation can cause similar albeit weaker effect on PDMS surface as oxygen plasma treatment (Qiu, Wu et al. 2014). The azide group is activated at the same time via the UV-irradiation causing the crosslinker to immediately bind to the PDMS surface, leaving the amine reactive NHS-ester side pointing outwards ready to be utilized in covalent binding of collagen. Interestingly, the concentration of sulfo-SANPAH did not seem to affect the amount of bound collagen, but the cell behaviour. Low concentration inhibited cell spreading and caused terminal differentiation; whereas higher concentration kept them undifferentiated, while letting them proliferate and spread out. (Trappmann, Gautrot et al. 2012)

At the time of the study by Trappmann *et al*., the usefulness of azido compounds in PDMS surface treatment methods was not, however, a new concept. As demonstrated above by Nishikawa et al., Gomez *et al.* in a similar fashion created a neural growth factor treated grooved PDMS surface for supporting embryonic hippocampal neuron culture and axon development. Their method for surface treatment included three steps. First, polyallylamine (PAA), a polymer with amine group in every monomer, was conjugated with n-4-(azidobenzoyloxy)succinimide (ABS), which is another molecule with phenyl azide and NHS-ester functional groups. The NHS-ester side of ABS binds into the amine of PAA leading into azido group terminated branched polymer structure. As a second step the PAA-ABS is cast and physisorbed twice on PDMS and dried. The third and final step includes the addition of neural growth factor solution and UV-irradiation. The UV-irradiation activates the azides, while also causing some functionalization of PDMS surface similarly as in the later study by Trappmann *et al*. (Trappmann, Gautrot et al. 2012). The

azides in PAA-ABS bind to both the amines in the growth factors and the PDMS surface, covalently immobilizing the neural growth factors on the PDMS. (Gomez, Lu et al. 2007)

LBL methods are also popular. In the studies by Brown *et al.* and Wang *et al.*, for example, a polyethylene imine and polystyrene sulfonate based LBL coating was used in similar fashion as by Wipff *et al* in the study mentioned previously in this Section. Brown *et al.* were able to show that vascular smooth muscle cells can be grown on LBL treated PDMS even without any cell adhesion molecules; with comparable results to standard tissue culture plastic. According to the study, LBL treated PDMS supported cell culture better than physisorbed fibronectin coating. They also showed that decreasing the stiffness of the PDMS substrate significantly increased the cell proliferation (Brown, Ookawa et al. 2005). Wang *et al.* on the other hand report much lower CACO-2 cell numbers on LBL treated PDMS surface than on standard tissue culture plastic; an indication that cell type could have a major effect for the results (Wang, Sun et al. 2010). Chien *et al.* introduced a photoactivated electrostatic LBL method for various substrates, including PDMS. They introduced alternating layers of polyacrylic acid and polyacrylamide on oxygen plasma treated PDMS. After several bilayers, there would be one azido functionalized polyacrylic acid layer. The LBL process can be continued until the desired thickness is achieved. Afterwards, when the layers are treated with UV-light, the interwoven polymers with azido groups bind the layers together. As many times before, the azido functionalized polymers are further functionalized with cell adhesion molecules or cell adhesion inhibitor molecules. The binding via azido groups happens only through layers that are exposed to the UV-irradiation. By using custom designed photomasks, it is possible to create micropatterned cell adhesive or inhibitive areas. The unbound parts of the polymer layers are washed away. Chien *et al.* showed that cells can attach and grow on the LBL treated surface, and that they will pattern along the grooves. One regular layer took slightly over ten minutes to create and one azido functionalized layer took 30 minutes, so the process can be lengthy, when creating thick LBL structures (Chien, Chang et al. 2009).

While this is not a complete review of all the research in the field, it shows the general trend in the current most useful surface treatment methods for PDMS in biomedical and cell culture research. In all of the studies introduced here, the authors had spent a great effort to understand the chemical reactions behind the various types of surface treatment methods. It is imperative to achieve comparable results, reproducible methods, and thus useful studies, because even after eliminating the unknown variables in the surface treatment process, the unpredictable nature of the cell culture still stands. While many of the chemicals are extremely predictable and usually efficient in their chemical nature, their realistic applicability for cell culture, for example, is a huge question mark. Chemicals with crosslinking capability are usually extremely cytotoxic, and any unbound or unreacted molecules can act as unspecific fixatives to the delicate cellular organelles. In addition, many of the tailor made crosslinkers are very expensive, which further limits their

application in large scale. There is a wide variety of interesting molecules with capabilities for crosslinking reactions that only wait to be exploited by adventurous researchers. As an example, Tiller *et al.* showed that ascorbic acid (AA), or vitamin C, has this type of capability and used it to bind enzymes to NH_2 functionalized surfaces (Tiller, Berlin et al. 1999). Despite its beneficial properties, ascorbic acid has not yet been utilized, however, in cell culture applications. This concept was taken further in this thesis work to create novel AA crosslinker based surface treatment methods specifically for cell culture. A multidisciplinary approach in the studies in this field is truly appreciated, if not essential.

EXPERIMENTAL PART

4. MATERIALS AND METHODS

To answer to the increasing need for basic research and more cell friendly solutions in PDMS based cell stretching applications, four PDMS surface treatment methods in seven variations were studied in static and dynamic conditions in this thesis work. The methods were based on either physisorption or covalent chemical bonding of collagen type I on PDMS. Physisorption was studied in two surface treatment variations PHY1 and PHY2 as well-founded references to the covalent methods. Covalent Method 1 with a single variation COGA found in literature (Wipff, Majd et al. 2009) utilizes GA crosslinking. In the study by Wipff *et al*, GA is used as a crosslinker between collagen type I and the substrate, immobilizing the protein on the PDMS surface and it offers a reference covalent method to the AA based methods developed in the thesis. The novel Covalent Method 2 with three variations COAA1, COAA2, and COAA3 utilizes AA based crosslinking as a solution to the possible cytotoxic effect of GA. Additionally, the results were used to propose and test Covalent Method 3, COGEL, that utilizes AA crosslinking and collagen type I gel.

The methods were studied in cell free environment with double fluorescent labelling to visually evaluate the amount of collagen type I bound on the PDMS surface. Covalent Method 1 was also imaged after two days of stretching and compared to physisorption methods. In addition to the cell free studies, Covalent Methods 1 and 2 were tested in human adipose stem cell (hAdSC) culture for 14 days in static conditions, and for 13 days in dynamic conditions to evaluate their suitability for extended dynamic culture. In the final part of the study, Covalent Method 3 was preliminary tested in cell free environment with Dulbecco's phosphate buffered saline (DPBS) for nine days and with static hAdSC culture for four days. The study was conducted in cooperation with BioMediTech, a joint institution of Tampere University and Tampere University of Technology, and as part of the Human Spare Parts and WoodBone projects. The cell culture work and live cell imaging were performed by Msc. Sanni Virjula of the Adult Stem Cell Group from BioMediTech.

This Chapter describes the basic information about the experiments done in this thesis work. Section 4.1 explains the manufacturing process of the CSDs and their counterparts, the static reference wells, from PDMS, while Section 4.2 explains how the stretching system works. The preparation of the different coatings can be found in Section 4.3. Chapter ends with Section 4.4 with the description of the fluorescent characterization of the coatings and the cell culture experiments.

4.1 PDMS device manufacture

Tailor-made CSDs from PDMS (Sylgard® 184, Dow Corning, MI, USA) and glass were applied in the study. The design and preparation of the devices, shown in Figure 9 have been described earlier by Kreutzer *et al*. (Kreutzer, Ikonen et al. 2013). The devices consist of a PDMS bulk, where a circular vacuum chamber and a cylindrical PDMS-based culture well are cut. The top of the device is sealed by a glass plate with a hole for creating the vacuum, and the bottom is sealed by a 120 µm thick PDMS membrane. The PDMS membrane provides the cell cultivation substrate. A small PDMS piece is added on top of the glass plate for mounting the tubing for vacuum. When a partial vacuum is created in the vacuum chamber, the elastomeric PDMS based culture well deflects, and deforms the cell cultivation substrate (Figure 10). The dimensions are illustrated in Figure 9.

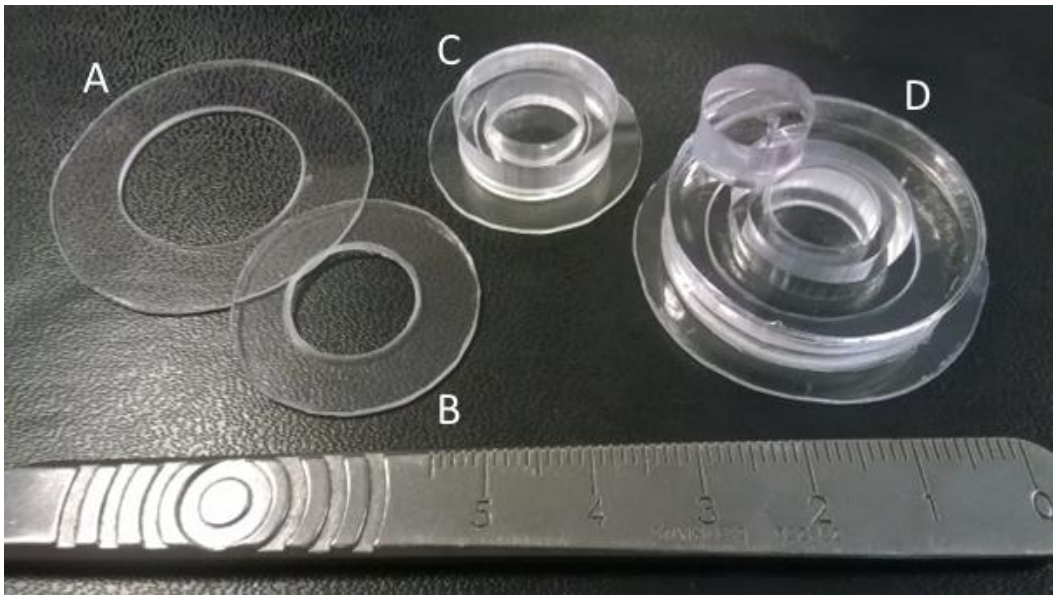

Figure 9. *The pneumatic CSD with PDMS membrane (D), static reference device with the same cell culture area (C), and separate polycarbonate rings of the devices (A and B) for preventing PDMS membranes from touching the surface below. The culture well is 12 mm in diameter (1.13 cm²) while the whole CSD is 31 mm wide.*

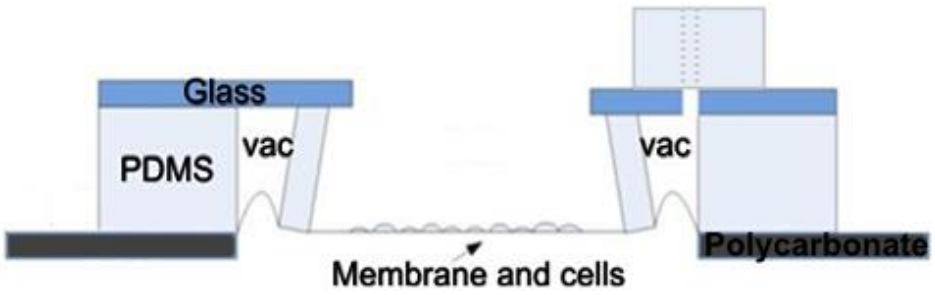

Figure 10. *Visualization of the cross section of the CSD and the vacuum applied stretch mechanism.*

Curing of the PDMS was done uncovered in an oven (Binder GmbH, Tuttlingen, Germany) in 60 °C for 10 hours to reduce the possibility of the ethylbenzene solvent residue. The individual pieces were treated with oxygen plasma (Pico-SR-PCCE, low pressure plasma system, Diener Elect., Ebhausen, Germany) to bond them permanently together. The plasma cleaner parameters were the same in all treatments (power 30 W; treatment time 20 s; chamber pressure 0.30; gas flow rate 1.4 sccm). The static reference wells which were used for unstretched cell culture experiments were prepared with the same dimensions for the cell culturing well as CSDs. The vacuum chamber was removed from the reference wells to save manufacturing costs and time (Figure 9).

All devices were mounted on specifically developed polycarbonate rings in order to prevent the PDMS membrane touching the surface under the devices. The polycarbonate rings for the reference wells had an outer diameter of 25 mm and an inner diameter of 13 mm. The rings for CSDs have an outer diameter of 40 mm and inner diameter of 20 mm. All rings were 0.8 mm thick.

The PDMS membrane was manufactured using the same PDMS curing protocol as for the devices. In order to attach the membranes to the devices, the membranes were first cleaned with isopropanol and deionized (DI) water, after which they were treated with oxygen plasma and bonded to the devices.

4.2 Cell stretching system

The vacuum stretching system was similar to the one used by Kreutzer *et al.* (Kreutzer, Ikonen et al. 2013) and it is composed of a laptop computer, LabVIEW-based controller software, a measurement board (National Instruments, USB-6229 BNC, USA), a computer controlled pressure regulator (T-2000, Marsh Bellofram, USA) attached to a high pressure outlet, and an ejector pump (Festo OY, VAD-1/8, Finland), which creates the vacuum. The CSDs on petri dishes were placed inside a cell culture incubator and attached to the ejector pump outside the incubator using a silicone rubber tubing system. The stretching was conducted under standard cell culture conditions in a humidified atmosphere (+37 °C, 5 % CO_2). Cyclic equiaxial stretching strain magnitude for the cell free durability test was 10 % with cyclic (sine wave, 0.5 Hz) stretching. Stretching for the cell culture test was applied with an effective stretching period of 12 hours per day followed by 12 hours of rest. The strain magnitude was increased from 2 % at the first stretching period to 3.5 % at the second period and finally to 5 % for the rest of the dynamic culture test.

4.3 Collagen type I coatings on PDMS

The membranes of the devices were functionalized with type I collagen from rat tail (Invitrogen, Life Technologies, Carlsbad, CA, USA) using different methods in five experimental phases. In phase one (P1), two physisorption methods and Covalent Method 1

were studied and imaged in cell free static and dynamic tests. In phase two (P2), Covalent Method 1 and 2 were imaged in cell free static test. In phase three (P3), one physisorption method and Covalent Method 1 and 2 were studied in static cell culture test. In phase four (P4), Covalent Method 1 and 2 were studied in dynamic cell culture test. In phase five (P5), Covalent Method 3 was proposed and imaged after the coating preparation. It was also preliminary studied in static cell culture test.

In this study, all the coated samples were used for characterization and cell culture within 24 hours of the coating preparation. For the cell culture experiments, the devices were first wiped outside with 70 % ethanol, then placed in a laminar hood and finally sterilized under mild UV-irradiation for 20 min. Finally, they were rinsed once with DPBS and placed in sterile cell culture plates.

4.3.1 Physisorption methods

PHY1 and PHY2 were prepared with one difference between them. PHY1 was left without plasma treatment while PHY2 samples were treated with oxygen plasma. All samples were incubated at room temperature for 60 minutes with collagen solution (50 µg/ml; diluted in 0.02 M acetic acid). The volume used to treat the wells contained 17 µg/cm² of collagen type I. Following the incubation, the samples were washed thoroughly with deionized (DI) water, dried for 15 minutes in a ventilation cabinet, and stored at +4 °C.

4.3.2 Covalent Method 1 with glutaraldehyde crosslinker

For Covalent Method 1, COGA, oxygen plasma, APTES (Sigma-Aldrich, St.Louis, MO, USA), and GA (Sigma-Aldrich) were used to covalently bond collagen type I onto the membrane (Wipff, Majd et al. 2009). After the plasma treatment, the membrane was treated with 10 % APTES solution in methanol for two minutes at room temperature to create a monolayer with primary amine groups on top. The samples were then washed once with methanol and rinsed twice with DI water. Then the COGA samples were treated with 3 % GA solution in DI water for 20 minutes and rinsed five times with DI water. Finally, the devices were treated with collagen type I as in the PHY coatings. Figure 11 illustrates the stages present in COGA method.

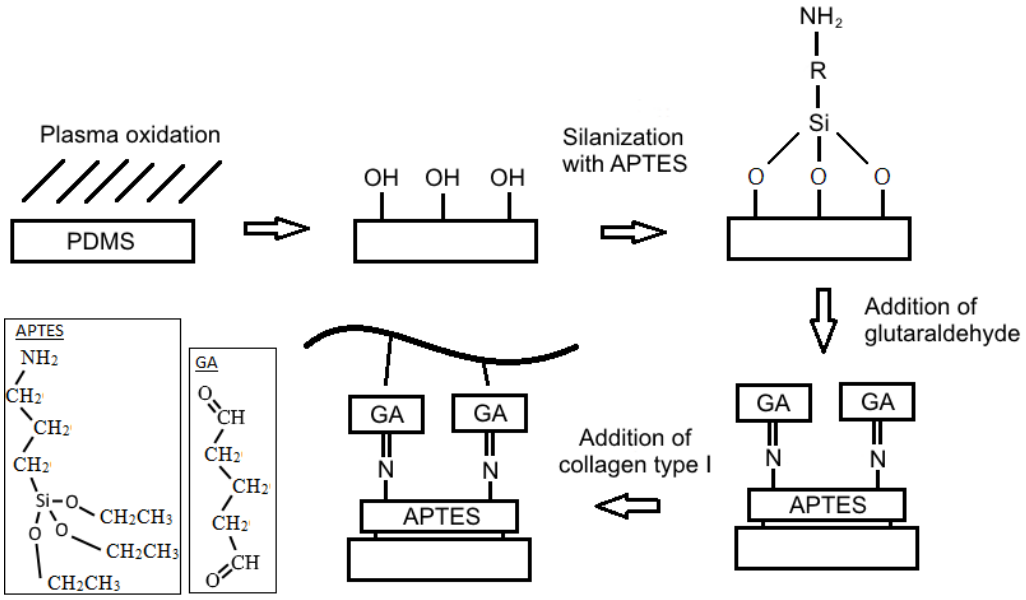

Figure 11. *A general illustration of Covalent Method 1 and the crosslinking mechanism between APTES, GA and collagen type I.*

4.3.3 Covalent Method 2 with ascorbic acid crosslinker

Covalent Method 2 was divided into three variations. The COAA1, COAA2 and COAA3 samples were prepared identically to the COGA samples, except the GA as the crosslinker was replaced by AA (Sigma-Aldrich, cell culture grade, ≥98 %) and the incubation time was prolonged from 20 minutes to 60 minutes followed by only two washings with DI water. For the COAA1 coatings, the AA powder was dissolved in DPBS (200 mg/ml; Lonza, BioWhittaker™, Verviers, Belgium), whereas for the COAA2 and COAA3 coatings the AA powder was dissolved in methanol (20 mg/ml). In the COAA3 coating protocol, 5 µl of 35 % hydrogen peroxide, which is 2 % of the used volume, was added to the AA coating solution in methanol to improve AA reactivity (Tiller, Berlin et al. 1999).

4.3.4 Covalent Method 3 for collagen type I gel

Covalent Method 3, COGEL, was proposed and preliminary tested after the results from the previous phases. The COGEL samples were prepared identically to the COAA1 method until the collagen type I treatment. In COGEL, collagen type I was dissolved and diluted in methanol instead of acetic acid (50 µg/ml). To increase the effectiveness of the dilution, the container was stirred with pipette tip and warmed by hands until no visible collagen type I aggregates were seen. The samples were incubated with collagen type I-methanol solution at room temperature for 60 min. During the incubation period, most of the methanol evaporated leaving all of the collagen type I on the sample in a thick gel-like layer. The same washing and storing protocol from the other methods was used to finalize the treatment process.

4.4 Studying the coatings

4.4.1 Fluorescent characterization

The fluorescent characterizations were done to visualize collagen type I on the coatings. This would give information about how collagen is organized on the coatings, if at all. The fluorescent visualization of the coatings would also show, if they would change during stretching. All collagen coatings were characterized without cells by using immunofluorescent staining. Staining were done to visualize the collagen on the PDMS surface. In P1, two parallel pristine PDMS, PHY1, PHY2 and COGA samples were stained and imaged consecutively within 24 hours to refine the staining protocol. In continuation of the P1, two parallel pristine PDMS, PHY1, PHY2 and COGA samples were stained and imaged before (day 0) and after a stretching period of two days (day 2) to see the durability Covalent Method 1, COGA, under mechanical stimulation. As a reference, two parallel samples were similarly stained and imaged at days 0 and 2 without stretching. In P2, two parallel COGA, COAA1 and COAA2 samples were stained and imaged consecutively within 24 hours to visually evaluate Covalent Method 2 and the viability of the AA crosslinker. In P5, two parallel COGEL samples were stained and imaged immediately after staining (day 0), after day 3, and day 9 after being with DPBS in an incubator. Figure 12 visualizes the double fluorescent protocol utilized here.

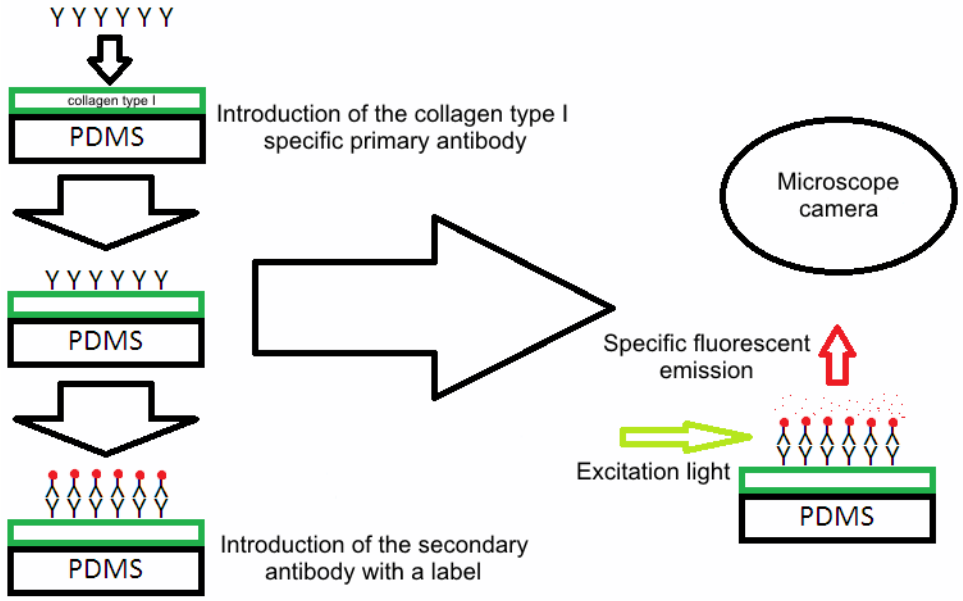

Figure 12. *Double fluorescent method for fluorescent imaging of the collagen type I coatings.*

All stainings regardless of the experiment phase used the same double fluorescent staining protocol. The staining protocol began with four quick washings using DPBS. After the washings, the unspecific bonding of antibodies was blocked using 1 % bovine serum al-

bumin (Sigma-Aldrich) diluted in DPBS. The blocking solution was incubated in the samples for 60 minutes at room temperature. Then, the coatings were incubated overnight at +4 °C with the anti-collagen type I primary antibody (ab90395, Abcam, Cambridge, UK) diluted 1:500 in the blocking solution. Next day, the devices were washed four times for three minutes with DPBS. The Alexa Fluor 488 ® conjugated secondary antibody (Life technologies) was diluted 1:800 in the blocking solution and the devices were incubated with the secondary antibody solution for 60 minutes at room temperature in dark. After the incubation, the devices were washed again four times for three minutes with DPBS and quickly rinsed once with DI water. The devices were imaged immediately after staining, or stored overnight at +4 °C in dark, and imaged latest in the morning. The greyscale and coloured images without cells were taken with the fluorescent microscope at Tampere University of Technology (Nikon Eclipse TS100 equipped for greyscale/coloured with Allied Vision Manta/Canon EOS550D cameras, Tokyo, Japan). The fluorescent images of COGEL coating in P5 were taken with Nikon Eclipse equipped with the Manta camera, and coloured in post processing with GNU image manipulation program. The relief images in P5 were created using the same program as well.

4.4.2 Adipose stem cell culture

The cell culture studies in P3, P4 and P5 were conducted in the facilities and in cooperation with the Adult Stem Cell Group from BioMediTech, and as part of the Human Spare Parts and WoodBone projects. All cell culture studies were also done in accordance with the Ethics Committee of the Pirkanmaa Hospital District. The hAdSCs were isolated from adipose tissue samples at Tampere University Hospital (Tampere, Finland). The cells of confirmed mesenchymal origin, the hAdSCs, were seeded onto pre-incubated static devices and CSDs. After the three-to-four-hour pre-incubation period, the incubation medium was first removed, 400 µl of fresh medium added and 1000 cells/cm^2 were seeded onto the samples in 100 µl of medium. Three parallel samples were used in static P3 and dynamic P4 culture tests. Static P5 culture test used two parallel samples. In all experiments, the culture medium was changed twice a week.

In the end of the static P3 culture test, the samples were imaged with light microscopy (Zeiss Axio Scope.A1 equipped with AxioCam MRc5 camera, Carl Zeiss, Oberkochen, Germany) after 14 days of culture. In the dynamic P4 culture test, the samples were imaged at days 3+0, 3+3, 3+7 and 3+10 with light microscopy. The P4 culture test included a three day pre-stretching culture period (day 3+0), where the cells were allowed to adhere to the devices, after which the stretching was started. In the end of the P4 culture test, the proliferation and the cell number was evaluated with CyQuant® (Thermo Fisher Scientific). In the end of P5 cell culture test at day 4, the cells were imaged with light microscopy to evaluate the cell attachment on COGEL and its suitability for cell culture tests in the future.

5. RESULTS AND DISCUSSION

This Chapter presents and discusses results of this work. A major part of this thesis work was the learning and optimization of the fabrication protocol for the CSDs and parts thereof. However, the largest part and the main aim of the thesis was to find suitable coating methods for the PDMS CSDs that; 1) make long term (> 48 hours) stem cell dynamic culture practical; 2) are easy and straightforward to implement in a limited laboratory setting. The experimental part of the thesis work was divided in five phases to portray the evolution of the experiments and the results from each phase are presented and discussed in chronological order during this Chapter.

5.1 Phase one: Fluorescent characterization of Covalent Method 1

The P1 was focused on the fluorescent characterization of Covalent Method 1 against the physisorption methods and pristine PDMS control. COGA, PHY1, PHY2 and the control samples were first characterized using fluorescent microscopy imaging in a simple setting to visualize the collagen type I on the surfaces of the devices, and to visualize any differences in the physical form of collagen. The samples were imaged following their preparation and staining with the collagen type I specific labelled antibodies such that the intensity, structure and distribution of the collagen on the substrate could be qualitatively analysed. P1 acted as a preliminary experiment for the more advanced phases that follow.

Figure 13 shows the difference in the fluorescent signal belonging to collagen type I between the four high-magnified images of the samples. The image shows that collagen type I can be labelled and seen from the substrate, and that pristine PDMS sample is dark, as expected. According to the image, the PHY2 sample has adsorbed more collagen than PHY1 due to the plasma treatment before the coating. The collagen appeared, however, aggregated when compared to the COGA sample which had an evenly distributed layer of collagen on the substrate. Figure 14 shows fluorescent images from the two-day dynamic experiment. COGA, PHY1, PHY2 and the control samples were imaged after staining at day 0, after the two-day period of incubation in medium in static and stretched conditions. Day 0 results are consistent with the previous results in Figure 13 except for PHY2 collagen aggregation being less prominent in half of the samples (2/4). Static incubation of two days did not visibly affect the coatings. The dynamic samples, however, show that the aggregated collagen in PHY2 samples has dropped off, because of the strain caused by the stretching substrate. If cells would have been living on top of the collagen in PHY2 samples, while they could have had adhered there, they would have disengaged with the collagen, which is unacceptable for any cell culture. COGA samples, however,

retained the same appearance, thus showing the superiority of the covalent immobilization of collagen when compared to physisorption.

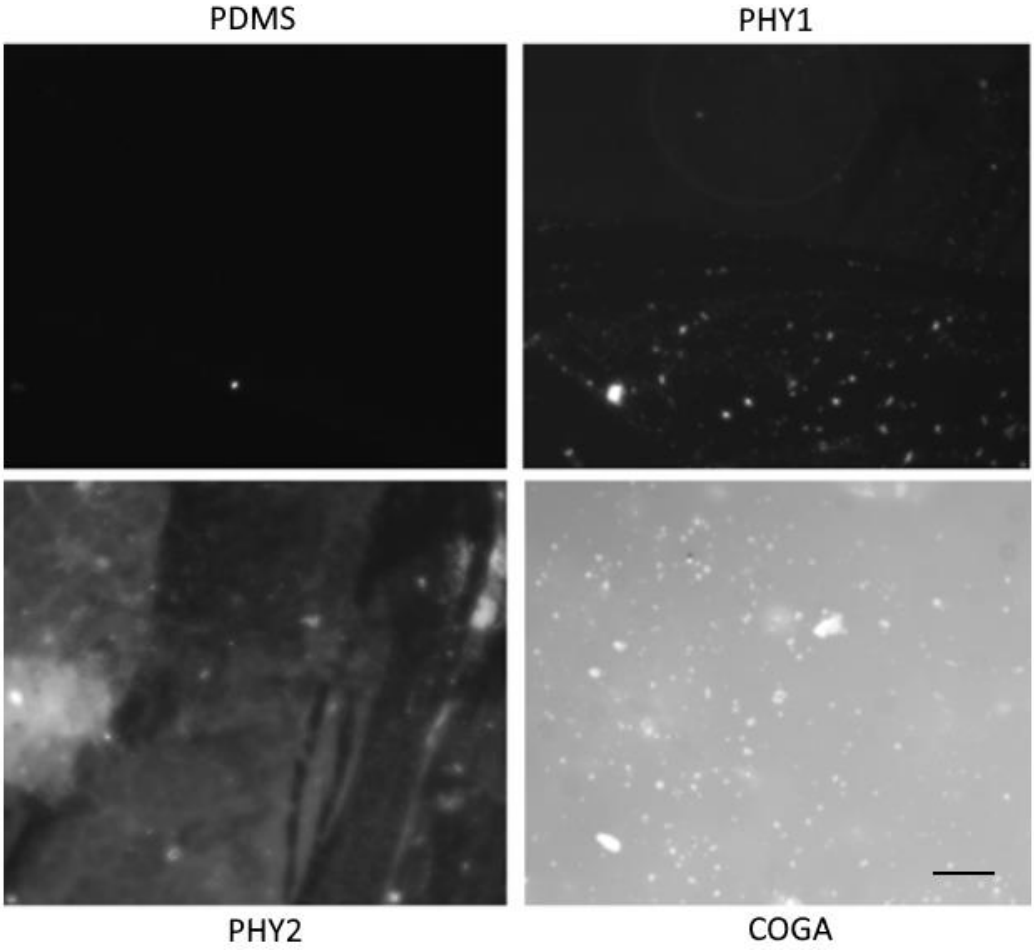

Figure 13. *High magnification immunofluorescent images of pristine PDMS well and wells coated with PHY1, PHY2, and COGA methods in P1. The images were taken using 20x (scale bar 50 µm) objective on Nikon Eclipse equipped with Manta camera.*

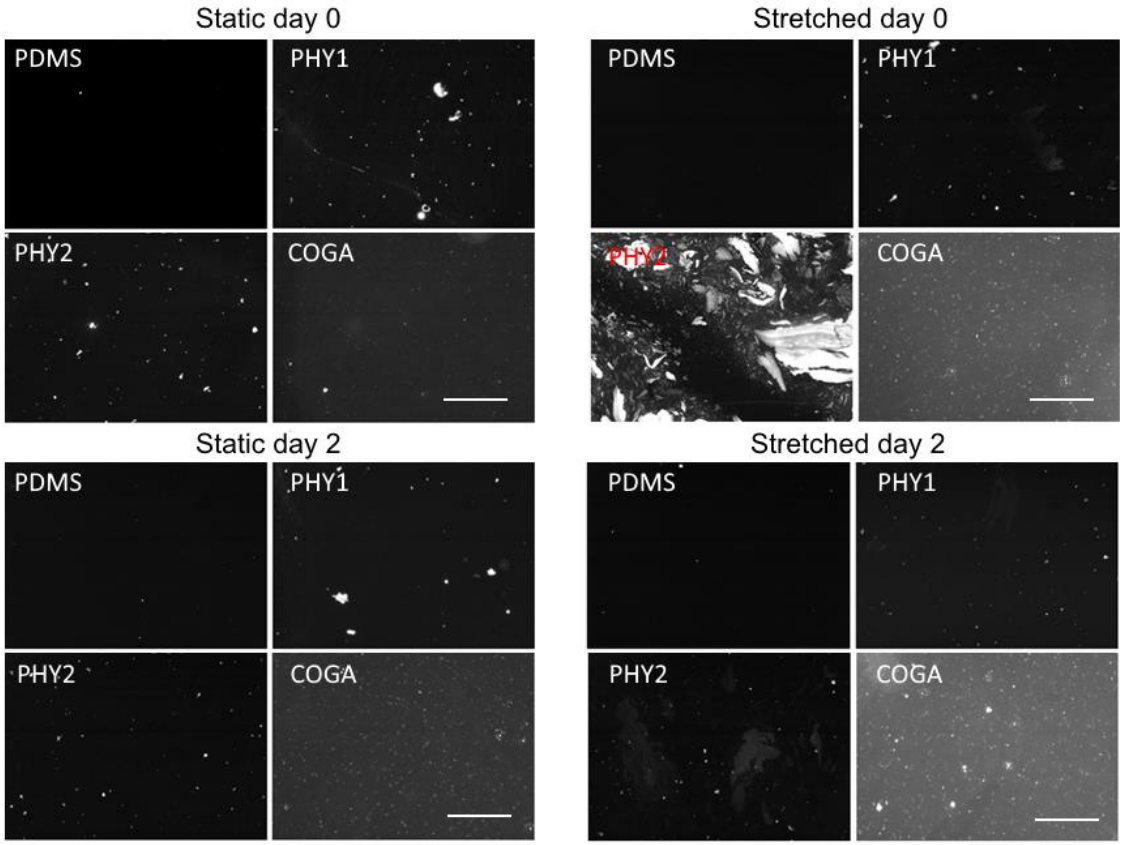

Figure 14. *Low magnification immunofluorescent images of pristine PDMS well and wells coated with PHY1, PHY2, and COGA methods in P1 after the two-day incubation period in static (left) and stretched (right) conditions. Images were taken using 4x (scale bar 500 µm) objective on Nikon Eclipse equipped with Manta camera.*

5.2 Phase two: Fluorescent characterization of Covalent Method 2

The Static experiments in P2 focused on the fluorescent characterization of the group of novel covalent methods that utilize AA crosslinker and their comparison with COGA that utilizes GA crosslinker. COGA, COAA1 and COAA2 were characterized by fluorescent microscopy similarly as in P1.

Figure 15 shows the images which strongly suggest superior binding capacity of AA crosslinker when compared to GA crosslinker. COGA maintains its evenly distributed layer of collagen also seen in P1; it can be better seen with longer exposure times or image post processing. However, COAA1 and to some extent COAA2 have completely different appearance. COAA1 method has bound a massive amount of collagen that in some areas seems to be in multiple layers; judged from the visible boundaries on the substrate. They resemble the aggregations of collagen on PHY2 which could mean that the collagen is

only physisorbed in large quantities and it will come off due to dynamic load or cell interaction. The fluorescent images show, however, that there is still an even collagen layer in both COAA1 and COAA2 between the aggregations. Both COAA1 and COAA2 look otherwise very similar when the largest collagen aggregations on COAA1 samples are disregarded. The stripes seen on the surface are likely on the PDMS substrate itself as the plasma oxidized thin PDMS membrane tends to form such stripes, likely due to the damaging effect of the oxygen plasma. These small cracks that form when the thin membrane deforms during manufacture, will then act as edges where molecules such as APTES, AA or collagen may accumulate and show on the images.

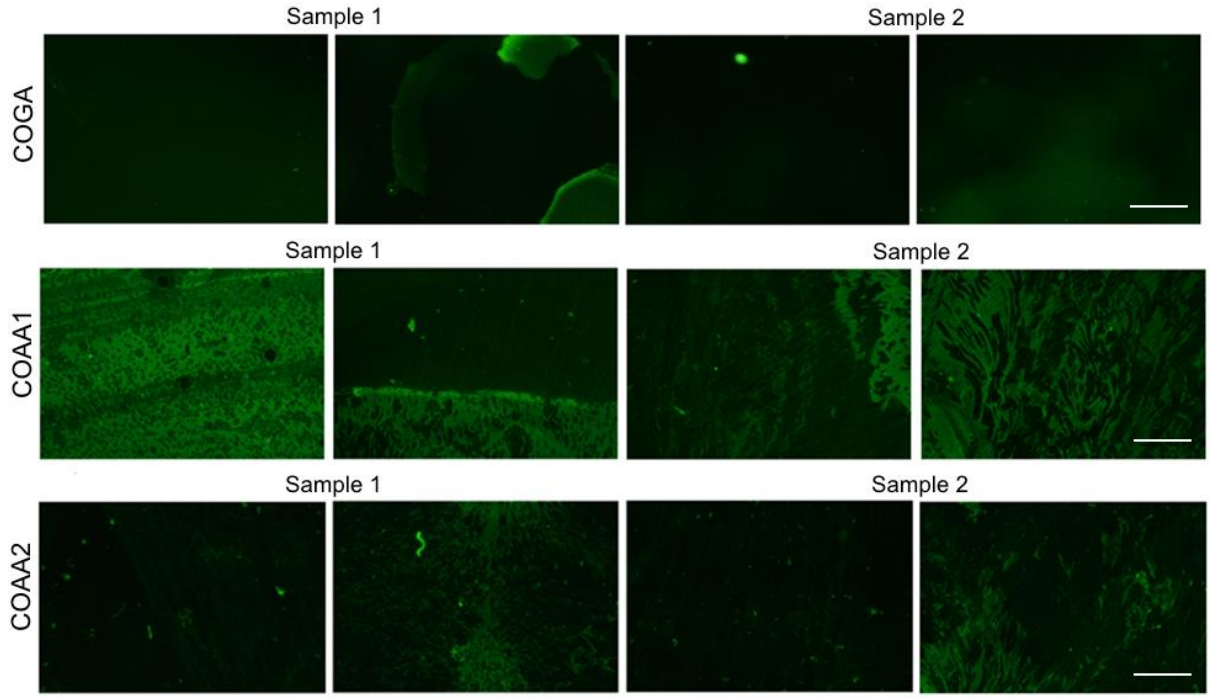

Figure 15. *Immunofluorescent images from two locations of two wells coated with either COGA, COAA1, or COAA2 methods in P2. Images were taken using 10x (scale bar 200 µm) objective on Nikon Eclipse equipped with Canon EOS550D colour camera.*

5.3 Phase three: Static adipose stem cell culture

P3 of the experimental part focused on growing hAdSCs for 14 days on three parallel PDMS static reference wells coated with PHY2, COGA, COAA1, COAA2 and COAA3 methods to validate their suitability for scientifically sound cell experiments. The cell cultures were followed and imaged using light microscope during and after 14 days of static culture and their viability and proliferation were qualitatively analysed.

Figure 16 shows the results of this experiment and differences between different coating methods are apparent. While seeded cells initially started to adhere on the PHY2 coated wells to some extent, along the way to day 14 almost all cells detached, leading to empty

substrate. This result tells that physisorption is unsuitable method for binding collagen on PDMS for long cell culture experiments. COGA coated wells fared better than PHY2 wells as the cells survived to the end of the experiment, adhered to the substrate, and started proliferating. They also created long processes and migrated on the substrate, which is typical behaviour for hAdSCs at this stage (Petrie, Doyle et al. 2009). However, by the day 14 the cell number had not raised much and the culture looked stagnant with debris from possibly dead cells all over the wells. The suspicion about the possible cytotoxicity of GA gains some credibility in light of these results. The experiments with AA crosslinker began with this issue in mind, and the aim was at minimum to increase the cell number. The COAA1 and COAA2 coated wells thrived when compared to the other methods. The COAA3 was comparable to the COGA which lead to questioning the cytotoxicity of the added hydrogen peroxide to the cells. The COAA3 method was added to the list, because of the evidence that it could lead to a more efficient crosslinking reaction for the AA (Tiller, Berlin et al. 1999). However, the cytotoxicity factor of hydrogen peroxide could have overweighed the positive effect of more efficient crosslinking. Similar effect was not seen in COAA1 and COAA2 wells, which provided excellent adhesion of the cells and fast proliferation that lead to near confluence at day 14.

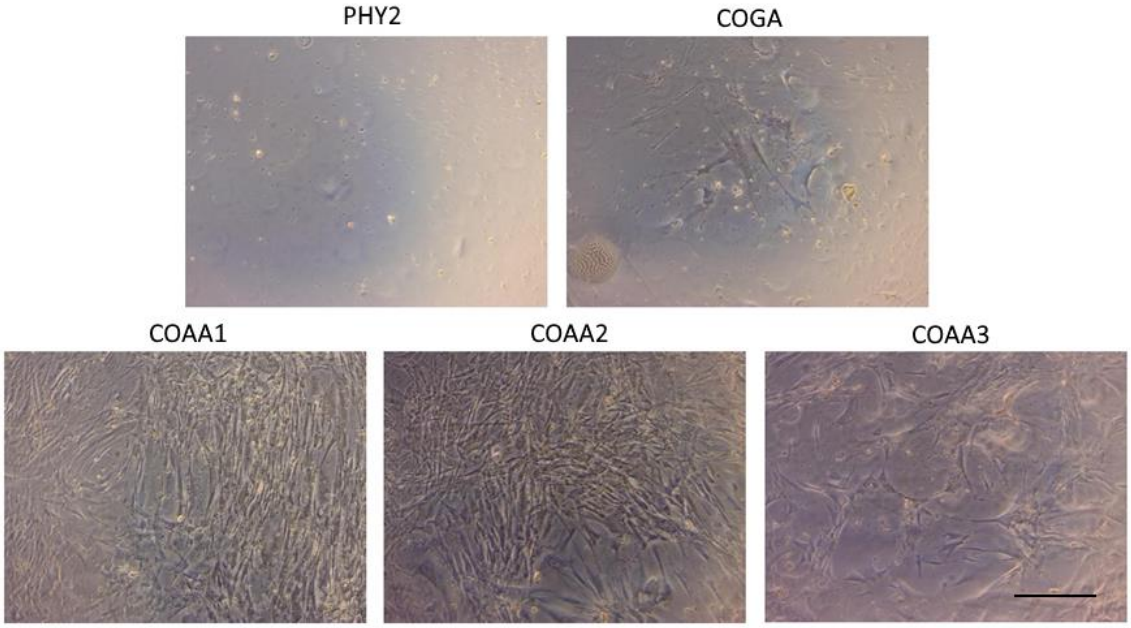

Figure 16. *Light microscopy images of hAdSCs grown for 14 days on static wells coated with PHY2, COGA, COAA1, COAA2, and COAA3 methods in P3. Images were taken using 5x (scale bar 200 µm) objective on Zeiss Axio Scope.A1.*

From these methods, COGA and COAA3 could support static hAdSC culture for 14 days, but with low proliferation and notable debris formation. Finally, COAA1 and COAA2 provided excellent support for static hAdSC culture, hence they have high potential for supporting also dynamic culture. PHY2 was unable to support static hAdSC culture for 14 days.

5.4 Phase four: Dynamic adipose stem cell culture

P4 of the experimental part of the thesis was the dynamic culture of hAdSCs on COGA, COAA1 and COAA2 coated samples. PHY2 and COAA3 were left out due to the insufficient results in P3 and to keep the sample number lower. The culture started as static for three days to give the cells time to adhere onto the substrate and continued as dynamic for 10 days for the half of the samples while the other half were kept static. The wells were imaged using light microscopy after 3+10 days and their cell number was quantitatively analysed with CyQuant®.

Figure 17 shows the wells at day 3+10. It is immediately apparent after looking at the images of the cells and the CyQuant® results in Figure 18 that COAA1 and COAA2 coated wells exhibit superior proliferation. Most of the COAA1 and COAA2 static wells had reached confluence, which is the reason of stopping the experiment at day 3+10 and not at day 3+11, which was initially planned. In one COAA2 sample, the layer of cells had started to peel off already, as it can be seen in Figure 17. The results are in line with the previous experiments in P3. In dynamic samples of COAA1, the cell number was at day 3+10 about 32 % lower than in static samples and in COAA2 the number was about 17 % lower. However, the COAA2 result could include some error as the cells in the static wells had begun to peel off, and therefore the cell number in the static sample could have been even higher. Overall, the cell numbers between COAA1 and COAA2 samples were quite similar. COGA samples, on the other hand, had significantly lower cell numbers at the end of the experiment. Unexpectedly, the static samples of COGA had lower cell number than the dynamic samples, further telling of the unexpected and random nature of COGA surface treatment method. However, this could also mean that the GA is able to immobilize collagen well to the surface and withstand the dynamic strain caused by the stretching keeping the cells attached to the substrate. This can cause the hypothetical cytotoxic effect of GA be more detrimental to the cell culture than the stretching itself. If this would be the case, the cell number disparity between static and dynamic samples would diminish.

In respect of the results in P4, COAA1 and COAA2 surface treatment methods for PDMS are suitable for dynamic hAdSC culture for 3+10 days. Due to the poor proliferation, the COGA method is not well suited for cell culture applications in neither static nor dynamic environment.

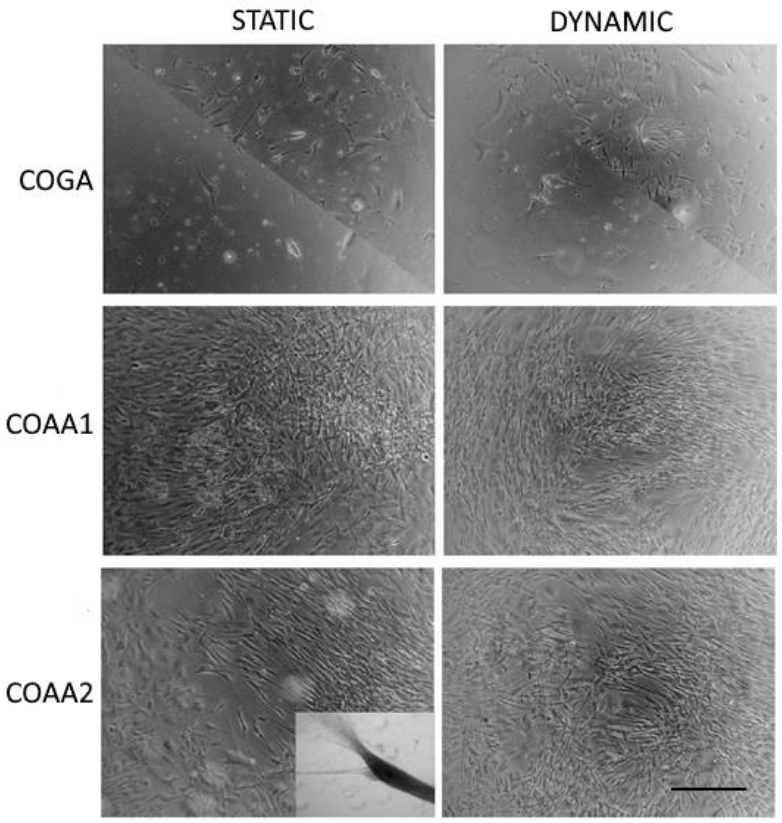

Figure 17. *Light microscopy images of hAdSCs grown for 3+10 days on static and dynamic wells coated with COGA, COAA1, and COAA2 methods in P4. Images of COGA samples show areas in the same well with different number of cells. The image of the static sample of COAA2 shows an area where the confluent cells had begun to detach. Images were taken using 5x (scale bar 200 µm) objective on Zaiss Axio Scope.A1.*

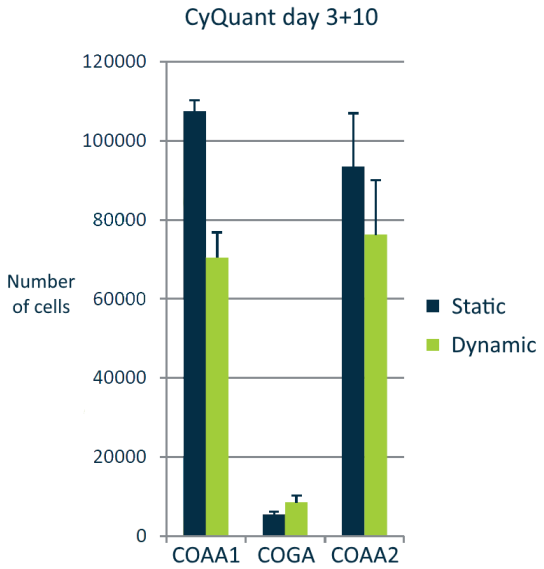

Figure 18. *The cell number of hAdSCs on static and dynamic wells coated with COGA, COAA1, and COAA2 methods in P4.*

5.5 Phase five: Preliminary tests with Covalent Method 3

In P5 of the experimental part, preliminary functionality tests were conducted with the COGEL coating, which was created with the results from P1 to P4 in mind. It was divided in two parts for visualizing collagen in cell-free culture environment, and for testing its suitability for hAdSC culture. The cell-free samples were imaged after day 0, day 3 and day 9. The cell culture samples were imaged after four days of culture.

5.5.1 Cell free incubation test

Figure 19 shows a low magnification image from a well with COGEL coating. The amount of collagen type I on the surface is very high, as expected. During the 60-minute incubation with collagen type I-methanol solution, the dissolved collagen fibrils had started aggregating into larger fibres, as it is apparent in Figure 19. Figure 20 shows a high magnification image of the coating, as well as a relief meant to highlight the 3D aspect. The areas in the samples showed varying amounts of collagen type I bundling. In some areas, there were short thin fibres (Figure 20, upper), and in some areas, there were clear matrix structures (Figure 20, lower). Multi-layered honeycomb-like collagen matrices, as seen here, can commonly be found in natural reticular connective tissue and bone, for example (Schwint, Labraga et al. 2004, Ushiki 2002), and it showcases collagen's ability for spontaneous fibrillogenesis without cellular influence. Figure 21 and Figure 22 show the samples from day 3 and 9 respectively. While some areas in the day 9 samples were slightly darker than in the others, there is not a big difference in the non-incubated day 0 samples and samples from day 3 or day 9. The presence of DPBS as a medium substitute and temperature of 37 °C did not greatly affect the coatings, which show easily identifiable fibrous and honeycomb-like structures even at day 9.

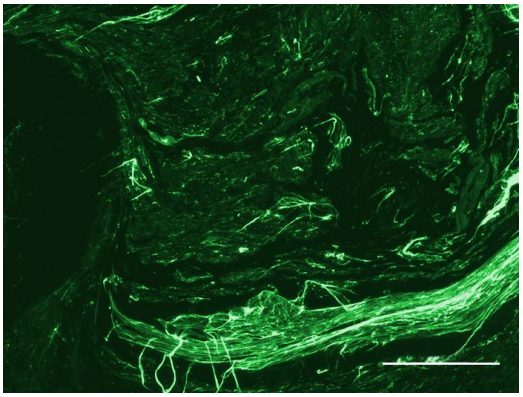

Figure 19. *Low magnification immunofluorescent image of the well coated with COGEL method at day 0 in P5. Image was taken using 4x (scale bar 500 µm) objective on Nikon Eclipse equipped with Manta camera.*

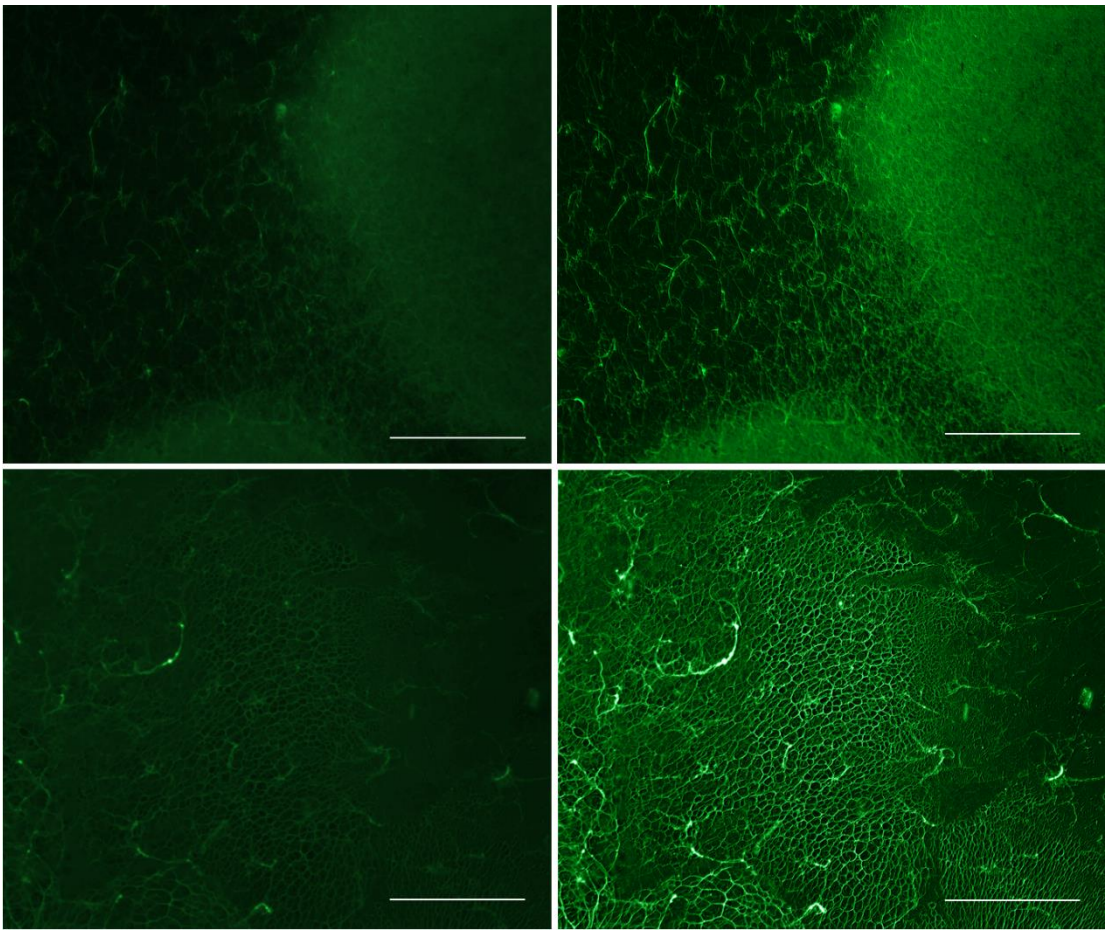

Figure 20. *High magnification immunofluorescent images of the well coated with CO-GEL method at day 0 in P5. The images on the left are shown as reliefs on the right. Images were taken using 10x (scale bar 200 µm) objective on Nikon Eclipse equipped with Manta camera. The reliefs were created using GNU image manipulation program.*

The results from the cell-free incubation test suggest that collagen type I takes a very robust form, when the protein is forcefully packed onto the substrate via evaporation of the solvent. In the other coating methods depicted in this thesis, collagen type I is only passively coming into contact with the substrate, which results into lesser amount of collagen available for fibrillogenesis, fibril formation. The result of this is most likely a coating that constitutes mostly of loosely bundled collagen fibrils that are structurally invisible in the scale of the images. When collagen is dissolved into a stock solution, it partially loses its fibrous formation. However, some of the natural crosslinks between the collagen alpha-helices are conserved, allowing the later self-assembly into larger fibrils and even fibres. Closely packed small fibrils that cannot organize into larger fibrils would likely be seen in the fluorescent images as smooth layers or structures; exactly what can be seen in the images from P2 (Figure 15). This lack of organization of the fibrils may result from the lower mobility and quantity of collagen available for bundling, as they are immobilized by the crosslinker on the substrate. When the collagen amount is increased, the less bundled surface collagen is buried under the excess, which then spontaneously crosslinks

with itself and forms larger fibrils. After the washing step, a gel layer visible to the naked eye formed on the substrate, an indication that the pores between the collagen fibres absorbed water and formed a collagen hydrogel. Collagen hydrogels are commonly used as scaffolds in tissue engineering applications and studies (Lee, Mooney 2001). While it is uncertain in light of these experiments, if the gel layer is covalently linked with the substrate, the results clearly show that the coating is resistant to incubation for nine days in physiological temperature and buffer solution.

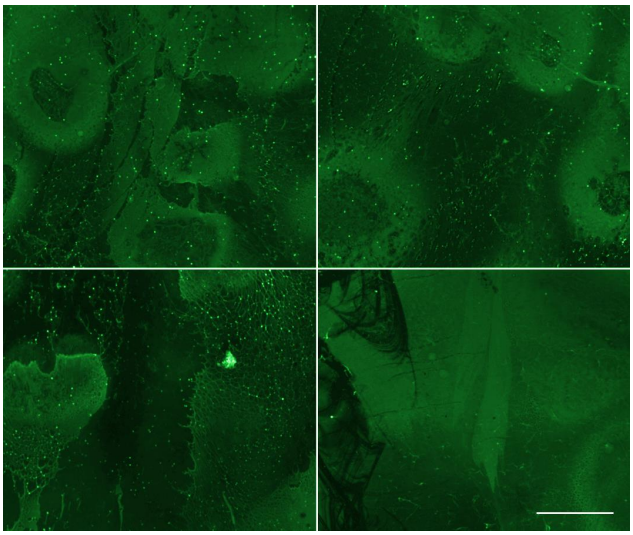

Figure 21. *Immunofluorescent images of the wells coated with COGEL method at day 3 in DPBS in P5. Images were taken using 4x (scale bar 500 µm) objective on Nikon Eclipse equipped with Manta camera.*

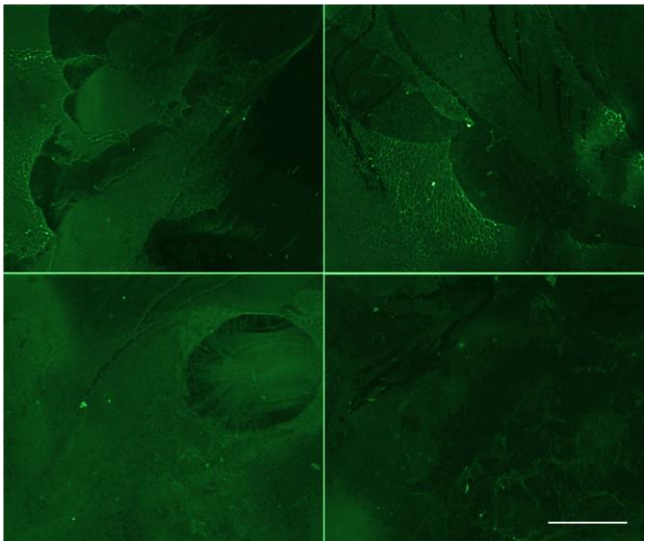

Figure 22. *Immunofluorescent images of the wells coated with COGEL method at day 9 in DPBS in P5. Images were taken using 4x (scale bar 500 µm) objective on Nikon Eclipse equipped with Manta camera.*

5.5.2 Adipose stem cell culture test

Alongside the cell-free samples, two COGEL coated samples were plated with hAdSCs similarly to the previous cell tests. The cells were cultured for four days and then imaged as seen in Figure 23. After day 4 of culture, the cells were well attached to the substrate and exhibited elongated processes from the main bodies, which is typical for stem cells of this type (Petrie, Doyle et al. 2009). The debris that is visible in the images was hypothesized to be small bubbles trapped inside the gel, as they seemed to be under the cells and did not seem to affect the culture negatively. While there was no control in this small-scale preliminary test, it can be said with reasonable certainty that hAdSCs can be successfully cultured on COGEL coated PDMS. It is also worthwhile to mention that after the culturing had been stopped and the cells removed from the incubator, the cells remained attached to the surface overnight, even though their morphology was already round, an indication of the incoming death. Nevertheless, while there are some clues about the durability of the new COGEL coating, there are still many uncertainties regarding the coating in a dynamic cell culture. For example, the amount of stretch that is transferred to the gel surface is unknown. These questions can only be answered by future experiments.

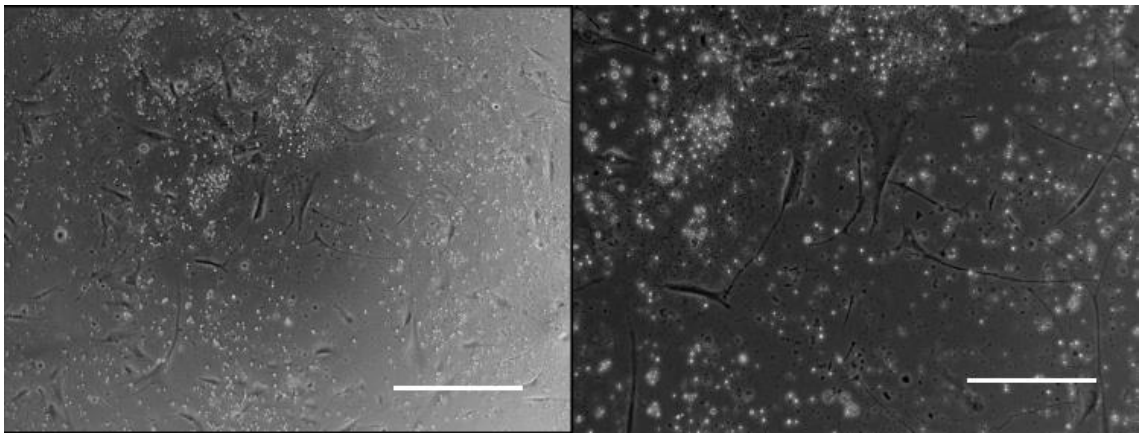

Figure 23. *Light microscopy images of hAdSCs grown for four days on static wells coated with COGEL method in P5. Images were taken using 5x (scale bar 200 µm) and 10x (scale bar 100 µm) objectives respectively on Zeiss Axio Scope.A1.*

6. CONCLUSION

The aim of this thesis was to find and apply surface treatment methods for PDMS that are suitable to be used in cell stretching applications, and to evaluate their capability to bind collagen type I and to support long term static and dynamic hAdSC culture. The motivation behind this study was to allow researchers in the Human Spare Parts and Woodbone projects to use the available custom made cell stretching system (Kreutzer, Ikonen et al. 2013) more effectively. The effect of stretching on cells has for a long time intrigued researchers worldwide and factors such as the method used for coating the device can have a significant effect on the end results.

The experimental study was divided in five phases that all focused on the various coating methods for PDMS membrane, which was a part of the CSD, and meant for cell culture. From these, the P1, P2 and P5 were fluorescent characterization experiments, while P3 and P4 were cell culture experiments. There were a total of seven different coating methods and a pristine PDMS control that were tested during the experiments of this thesis work; PHY1, physisorbed collagen type I on PDMS; PHY2, physisorbed collagen on plasma oxidized PDMS; COGA, immobilized collagen on APTES and GA; COAA1, immobilized collagen on APTES and AA in DPBS; COAA2, immobilized collagen on APTES and AA in methanol; COAA3, immobilized collagen on APTES and AA in methanol with added hydrogen peroxide; COGEL, immobilized collagen gel on APTES and AA.

P1, P2 and P5 showed that it is possible to label collagen on the substrates with a fluorescent dye and visualize its features with fluorescent microscope. The experiments in P1 also revealed that covalent binding of collagen by COGA is superior to physisorption methods especially after 2 days of stretching. In P2, COAA1 and COAA2 are in turn shown to bind superior amounts of collagen compared to COGA. P5 and the applied COGEL method showed that collagen type I can spontaneously organize into visible fibrils and honeycomb-like structures. The cell culture tests in P3 and P4 showed that COAA1 and COAA2 promoted cell adhesion and had superior proliferation of hAdSCs in all static and dynamic samples. COGEL also supported hAdSC attachment and proliferation in P5. In P3 and P4, COAA3 and COGA had both similarly low cell proliferation or high cell mortality rate, which could be a telling sign about the possible cytotoxicity of the used chemicals GA and hydrogen peroxide.

The main aim of this thesis work was to covalently bind collagen type I to PDMS CSDs for long-term cell stretching experiments. Then, if applicable, the secondary aim was to propose a novel surface treatment method to improve upon the existing methods. Both aims were achieved in this thesis work. The novel AA based Covalent Method 2 was successfully utilized in fluorescent imaging and cell stretching studies, improving the

binding of collagen and cell culture quality when compared to the popular physisorption method or the GA based Covalent Method 1. Covalent Method 2 managed to do so without complicating the treatment process or increasing the costs, thus proving to be a welcome addition to the repertoire of cell culture researchers. In addition, the coating methods developed in this thesis may improve functionalization efforts of tissue engineering scaffolds and implants with durable but cell friendly coatings. The world around the cells is constantly changing, but it is impossible to measure *in vivo* which specific forces affect the cells in which way, especially when speaking about the elusive stem cells. Studying dynamic cell culture is an important step in the search for the answers about the physical cues that affect stem cell differentiation and mature cell culture. The answers that can only be solved by rigorous basic research by tireless researchers worldwide.

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